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The Effects of Intrinsic and Extrinsic Factors on Neural Stem Cell Populations

Charlotte Elizabeth Scott

A thesis submitted to the University of London in fulfilment of the
requirements for the degree of Doctor of Philosophy.

April 2007

Division of Stem Cell Biology and Developmental Genetics
National Institute for Medical Research
The Ridgeway
Mill Hill London NW7 1AA

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I, Charlotte Elizabeth Scott, confirm that the work presented in this thesis is my own work.

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Dedication

To my Auntie, Fiona Stevenson, who first inspired me to work with neural stem cells.

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List of Abbreviations

| | |
|--------------------|---|
| β gal | β -galactosidase |
| β geo | β -galactosidase/neomycin fusion gene |
| CNS | central nervous system |
| $^{\circ}\text{C}$ | degrees Celsius |
| ddH ₂ O | double distilled water |
| DAPI | diaminido-2-phenylindole |
| dATP | 2'-deoxyadenosine 5'triphosphate |
| dCTP | 2'-deoxycytidine 5'triphosphate |
| dGTP | 2'-deoxyguanosine 5'triphosphate |
| dTTP | 2'-deoxythymidine 5'triphosphate |
| DNA | deoxyribonucleic acid |
| DMEM | Dulbecco's Modified Eagles Medium |
| DMEM-F12 | Dulbecco's Modified Eagles Medium Nutrient Mixture F-12 |
| DMF | N,N-Dimethylformamide |
| dpc | days post coitum |
| DT | dorsal telencephalon |
| DTT | dithiothreitol |
| E | embryonic day |
| EDTA | ethylene diamine tetraacetic acid |
| EGTA | ethylene glycol-bis (β -amino-ethylether)-N,N,N',N'-tetraacetic acid |
| ES cells | embryonic stem cells |
| FACS | fluorescent-activated cell sorting |
| bFGF | basic fibroblast growth factor |
| g | average acceleration |
| g | grams |
| HSC | haematopoietic stem cells |
| kb | kilobase |
| LB | Lurea broth |
| M | molar |
| MEM | MOPS, EGTA, magnesium sulphate |
| μg | microgram |

| | |
|------------|--|
| μl | microlitre |
| μm | micrometer |
| MOPS | 3-(N-morpholino) propanesulphonic acid |
| MSC | mesenchymal stem cell |
| NEC | neuroepithelial cell |
| <i>neo</i> | neomycin gene |
| NDM | neurosphere differentiation medium |
| NPC | neural progenitor cell |
| NPM | neurosphere proliferation medium |
| NSC | neural stem cell |
| OPC | oligodendrocyte precursor cell |
| PB | phosphate buffer |
| PBS | phosphate-buffered saline |
| PBS-A | phosphate-buffered saline (Dulbecco's solution A) |
| PBS-CMF | phosphate-buffered saline-calcium and magnesium free |
| PCR | polymerase chain reaction |
| PKA | protein kinase A |
| PNS | peripheral nervous system |
| RMS | rostral migratory stream |
| RNA | ribonucleic acid |
| RPC | retinal progenitor cell |
| RT-PCR | reverse transcription polymerase chain reaction |
| RT-PCR | real time polymerase chain reaction |
| SC | spinal cord |
| SDS | sodium dodecyl sulphate |
| SSC | standard saline solution |
| SVZ | subventricular zone |
| T | Triton X-100 |
| Tris | tris(hydroxymethyl)aminomethane |
| U | units |
| V | voltage |
| VZ | ventricular zone |
| w/v | weight to volume |
| X-gal | 5-Bromo-4-chlor-3-indoyl-β-D-galactopyranose |

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Abstract

Neural stem cells (NSCs) are uncommitted cells of the central nervous system (CNS), defined by their ability to self renew and to generate neurons, astrocytes and oligodendrocytes. These cells contribute to the formation of the CNS during embryonic development and in adults for tissue homeostasis. Currently, the best *in vitro* assay for NSCs is their ability to clonally form neurospheres. Neurospheres are free-floating spherical structures, composed of NSCs, neural progenitor cells (NPCs) as well as differentiated neural cells.

To better understand the formation and regulation of NSCs, the developmental time points at which neurospheres can form from the mouse CNS were determined. Novel culture conditions for chick neurosphere culture were then defined and used to characterise the stages neurospheres can be generated from chick CNS tissue.

Second, the role of *Sox9* in neurosphere formation was investigated. This is a member of the class E group of HMG box containing SOX transcription factors and studies have shown it to be a vital component of the neuron-glia switch. In recent years, radial glia have been reported to exhibit NSC-like properties. Evidence is provided that SOX9 expression coincides with the timing of neurosphere formation from the mouse CNS. Moreover, gain and loss-of-function experiments indicate *Sox9* is both necessary and sufficient for neurosphere formation. In addition, *Sox9* was able to significantly increase neurosphere generation from the adult SVZ.

The secreted molecule sonic hedgehog (SHH) has been implicated in promoting NSC formation and growth. In support of this, experiments described in this thesis suggest that SHH increases both the number of neurosphere forming cells and the proliferation of cells within the neurosphere in E11.5 mouse spinal cord cultures. Experiments using neurospheres derived from embryonic chick spinal cord and telencephalon

cultures reveal a similar proliferative response to SHH as mouse CNS cultures, suggesting conservation of molecular mechanisms. In addition, evidence is provided that SHH can induce early neurosphere formation, both in the embryonic mouse and chick. Moreover, SHH is shown to significantly increase neurosphere generation from the adult NSC niche, the subventricular zone (SVZ).

Finally evidence is provided that *Sox9* acts downstream of SHH, establishing a functional link between extrinsic and intrinsic factors that control NSCs.

Chapter 1

Introduction

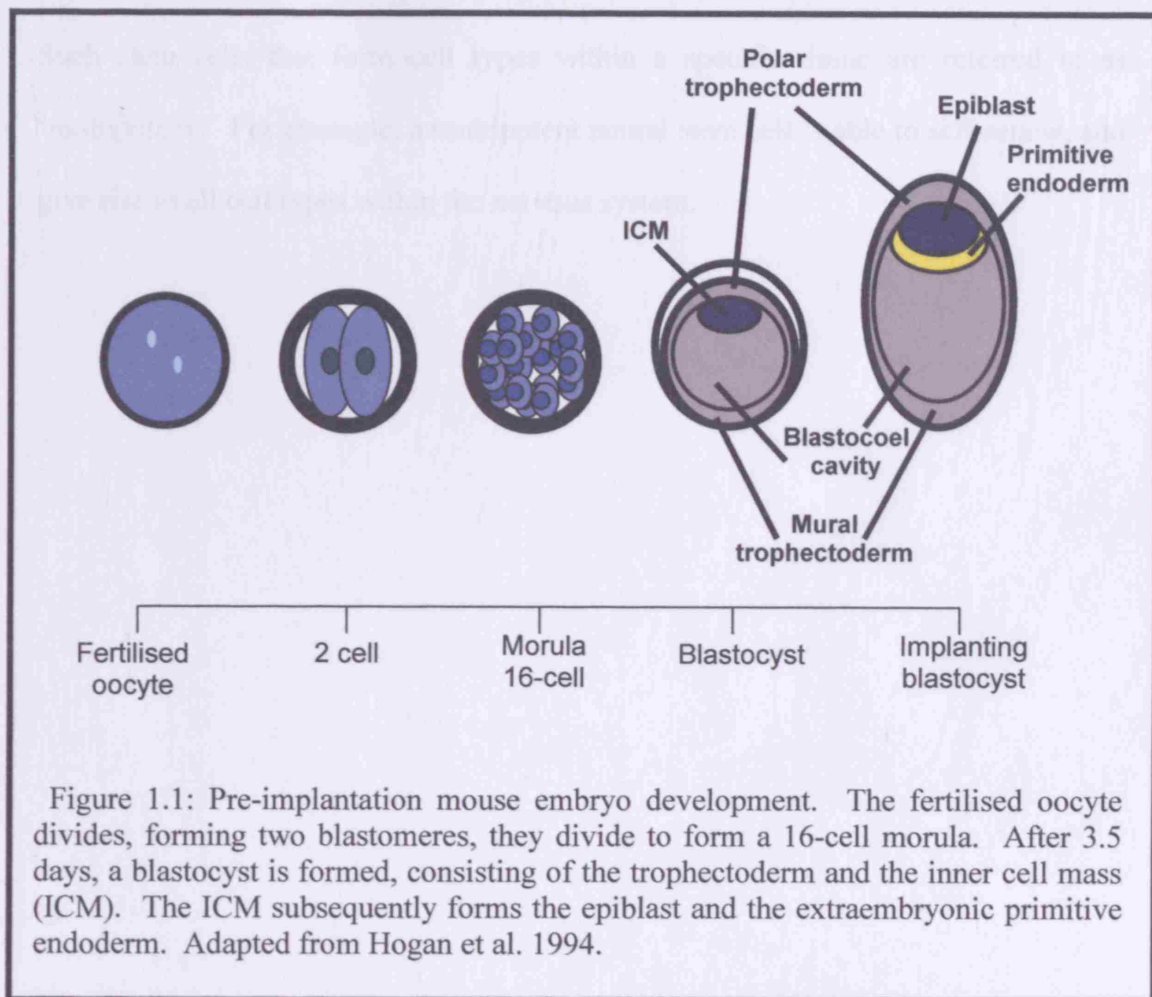
Chapter 1

Introduction

1:1. Stem Cells

Stem cells are defined by their ability to self-renew, as well as give rise to various cell types. Stem cells are subdivided based on their potential to generate differentiated cell types. During early development, the fertilised mouse egg undergoes several cleavage divisions, giving rise to two, then four, then eight apparently identical daughter cells, termed blastomeres (Howlett, 1985). Each of these blastomeres is capable of generating all specialised cell types within the organism, and can therefore be termed ‘totipotent’. The blastomeres continue to divide, eventually forming a blastocyst, composed of outer trophectoderm cells, fated to give rise to the various trophoblast cell types of the placenta, and the inner cell mass (ICM). The ICM cells are able to give rise to other extraembryonic tissues as well as to all cell types in the resulting animal (Figure 1.1). Although they have significant potential, cells of the ICM are no longer able to give rise to trophectoderm or to organise themselves into a proper embryo. They are therefore referred to as ‘pluripotent’ rather than totipotent.

The ICM undergoes a further round of cell specification, giving rise to extraembryonic endoderm and to the epiblast. The epiblast will contribute to the embryo proper during subsequent development (Gardner, 1985; Gardner, 1979), and the endoderm remains restricted to the extraembryonic parietal and visceral endoderm in the yolk sac surrounding the embryo (Gardner, 1982; Gardner, 1984).



During gastrulation, the three primary germ layers, the ectoderm, endoderm and mesoderm are determined. The formation and position of a transient embryonic structure, termed the primitive streak, defines the anterior-posterior axis of the future embryo (Tam and Behringer, 1997; Beddington, 1999). The ectoderm will eventually give rise to the skin and the central nervous system, the endoderm to the lungs, gastrointestinal tract and associated organs, and the mesoderm to the skeleton, muscles, kidneys, gonads, connective tissue, bone, blood vessels, and blood forming tissue (Figure 1.2) (Gardner, 1983; Lawson, 1991; Tam, 1987; Wilson, 1996). The emergence of specialised cells is associated with progenitor cells adopting a more restricted potential. However, many differentiating or even adult systems retain a

population of uncommitted cells that, on division, can self-renew or differentiate. Such stem cells that form cell types within a specific tissue are referred to as 'multipotent'. For example, a multipotent neural stem cell is able to self-renew, and give rise to all cell types within the nervous system.

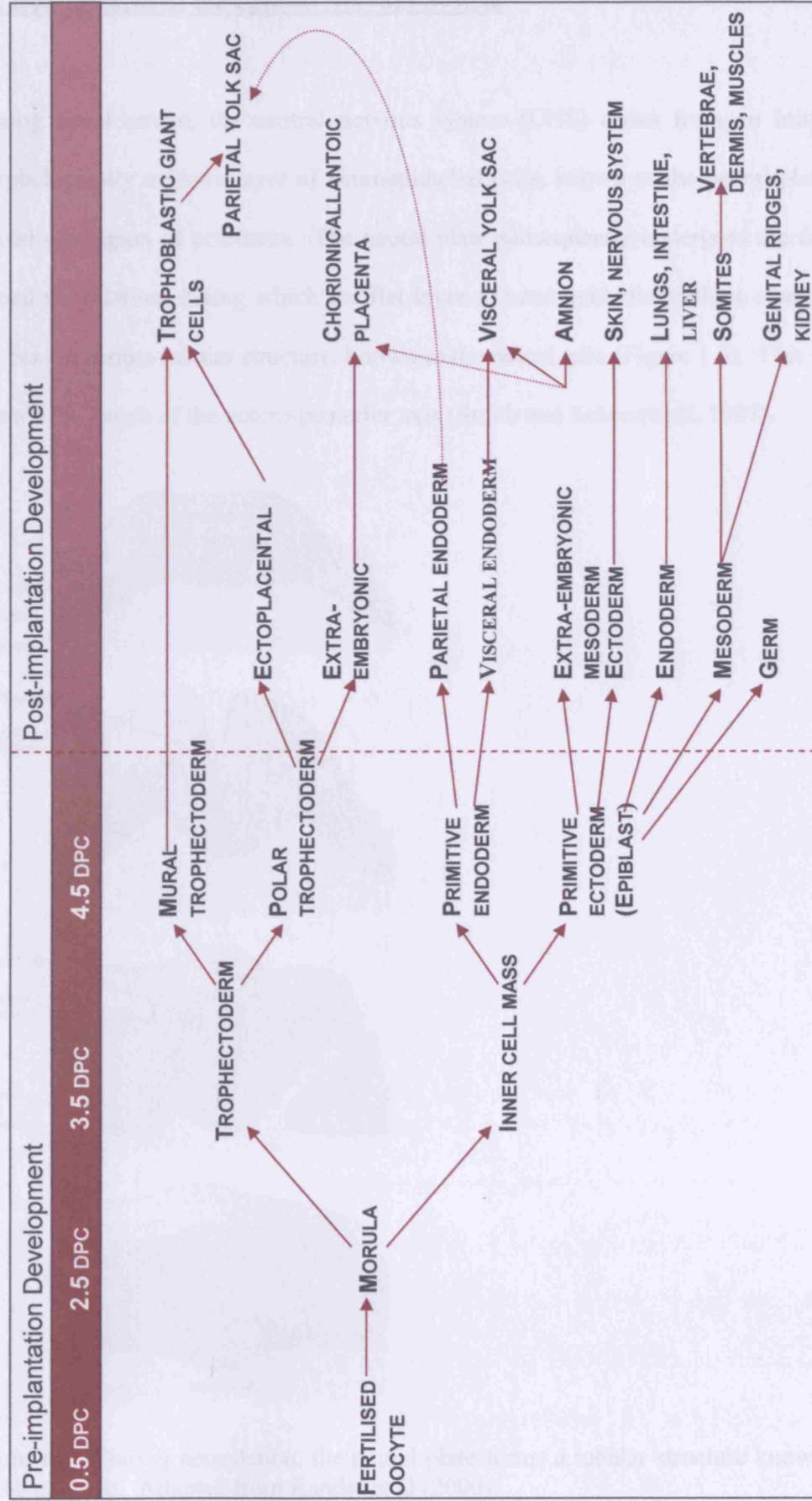


Figure1. 2- CELL LINEAGE RESTRICTIONS DURING PRE-IMPLANTATION AND POST-IMPLANTATION DEVELOPMENT. ADAPTED FROM GARDNER, (1982).

1:2. Development of the Central Nervous System

During development, the central nervous system (CNS) arises from an initially morphologically uniform layer of neuroepithelial cells, known as the neural plate, a specialised region of ectoderm. The neural plate subsequently undergoes a process termed neurulation, during which the flat layer of neuroepithelial cells is elongated and bent to form a tubular structure, known as the neural tube (Figure 1.3). This tube extends the length of the antero-posterior axis (Smith and Schoenwolf, 1997).

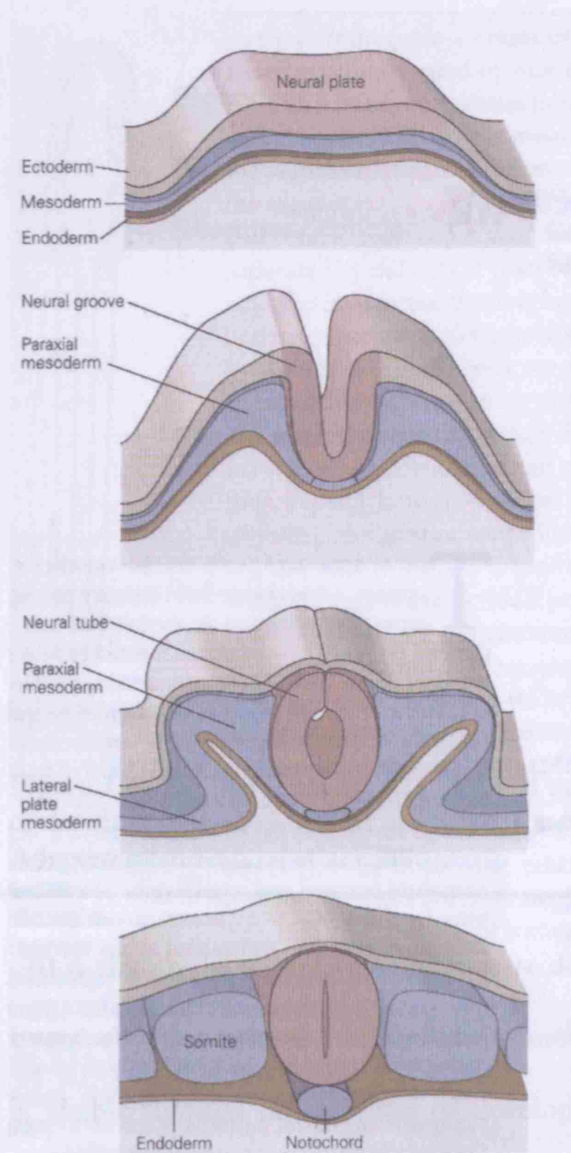
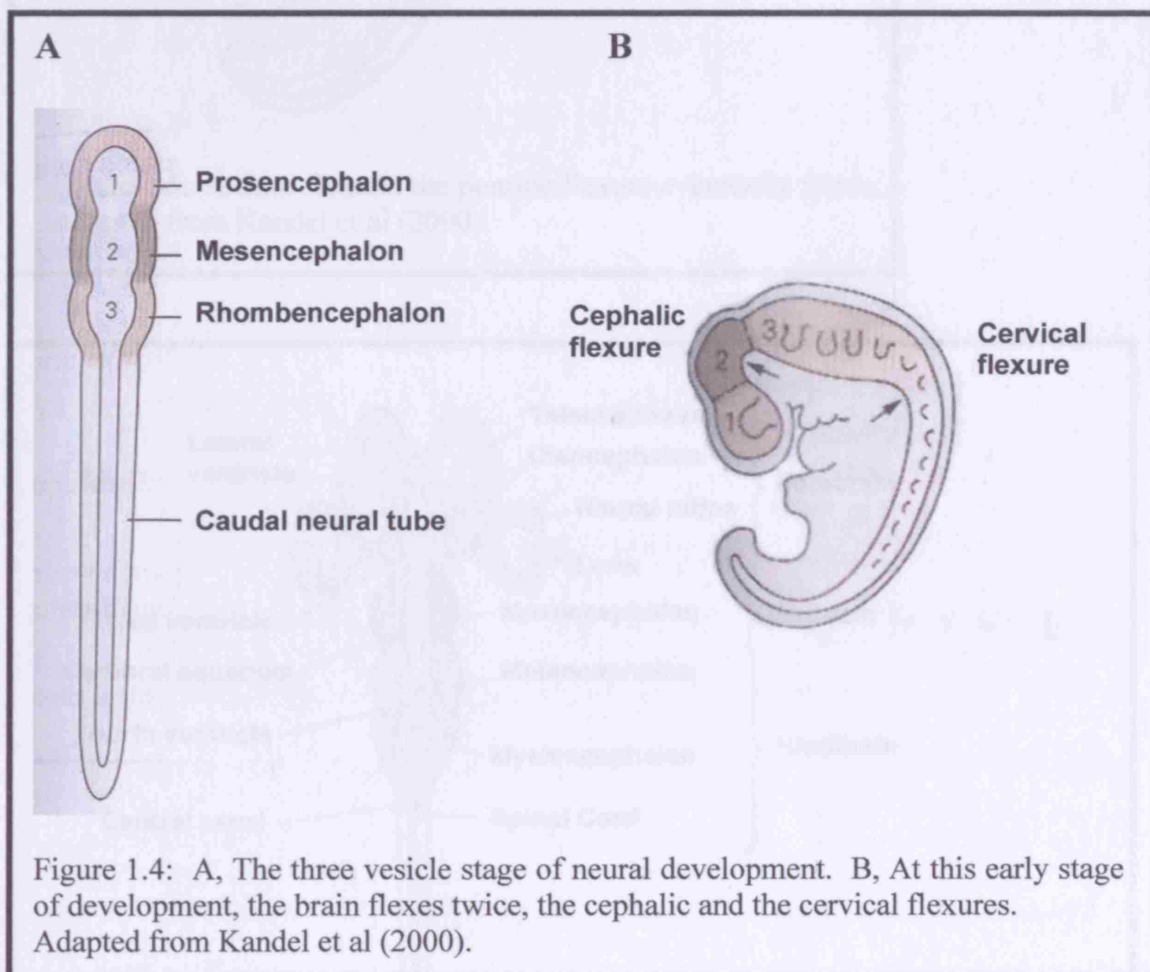


Figure 1.3: During neurulation, the neural plate forms a tubular structure known as the neural tube. Adapted from Kandel et al (2000).

This neural tube then begins to acquire rostro-caudal regionalisation. The caudal region will become the spinal cord, and the rostral region the brain. The brain is divided into three brain regions, the forebrain (prosencephalon), midbrain (mesencephalon) and the hindbrain (rhombencephalon) (Figure 1.4A). At this stage, the brain flexes twice (Figure 1.4B), the most caudal flexure, the cervical flexure, is at the junction between the spinal cord and hindbrain, the second is the cephalic flexure occurring between the hindbrain and the midbrain (Kandel et al. 2000).



At a slightly later stage a third flexure develops the pontine flexure (Figure 1.5). Eventually, the cervical and pontine flexures straighten, however the cephalic flexure is visible through the duration of development. As development progresses, the forebrain and hindbrain divide further, the forebrain forms the telencephalon and diencephalon, while the hindbrain gives rise to the metencephalon and

myelencephalon (Figure 1.6). Including the spinal cord, these regions make up the mature CNS.

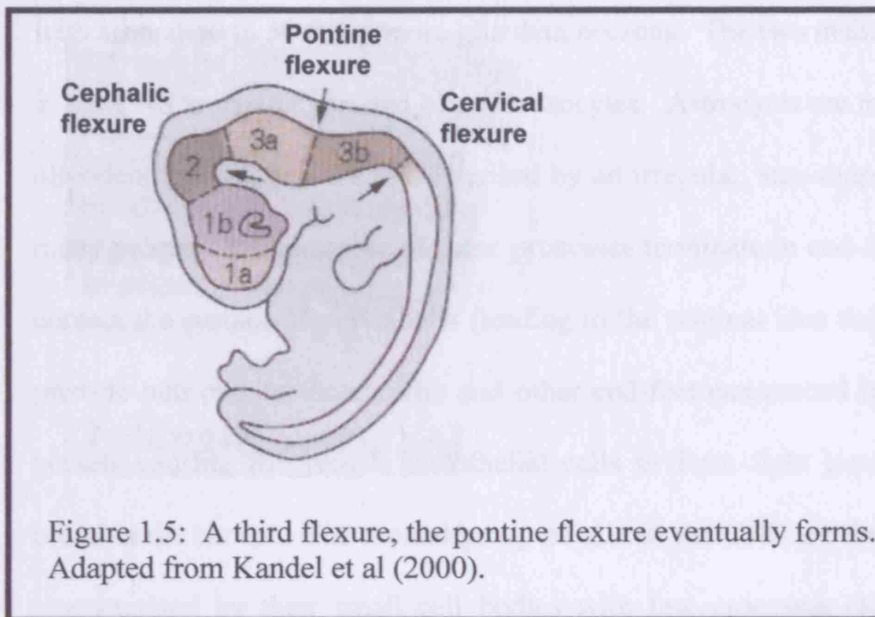


Figure 1.5: A third flexure, the pontine flexure eventually forms. Adapted from Kandel et al (2000).

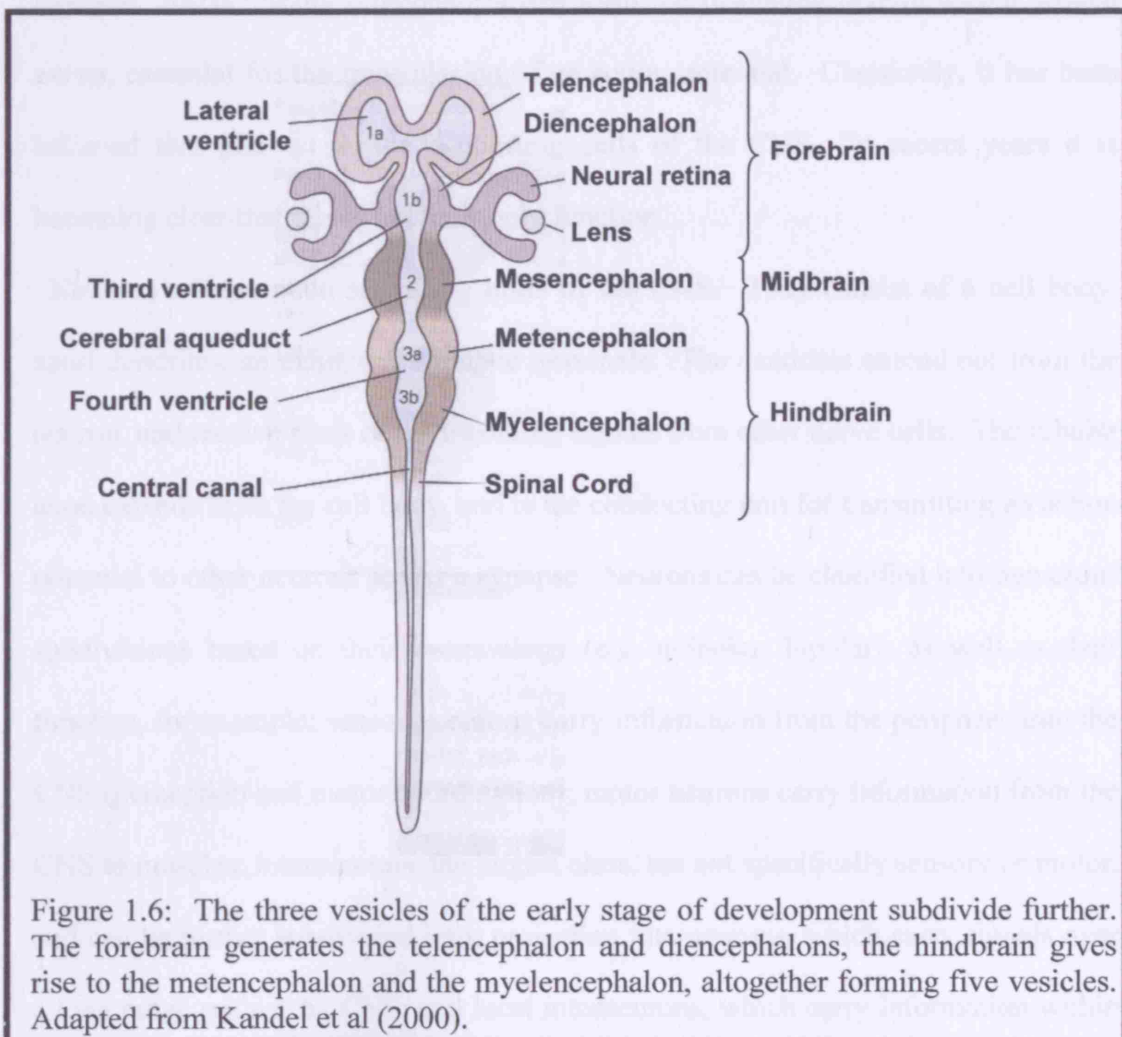


Figure 1.6: The three vesicles of the early stage of development subdivide further. The forebrain generates the telencephalon and diencephalons, the hindbrain gives rise to the metencephalon and the myelencephalon, altogether forming five vesicles. Adapted from Kandel et al (2000).

The position specific expression of developmental genes, determines the cell types that differentiate from the neural tube. In the CNS, glial cells outnumber neurons, with around 10 to 50 times more glia than neurons. The two main types of glial cells in the CNS are astrocytes and oligodendrocytes. Astrocytes are more numerous than oligodendrocytes, and are characterised by an irregular, star-shaped body with often many processes. A number of these processes terminate in end-feet, some of which contact the surface of nerve cells (leading to the original idea that astrocytes merely provide nutrients to these cells) and other end-feet can extend to the brain's blood vessels causing the vessels endothelial cells to form tight junctions, creating the blood-brain barrier. The second group of glia in the CNS are the oligodendrocytes, characterised by their small cell bodies with few processes (Kandel et al. 2000; Doetsch, 2003). Many oligodendrocytes form the insulating myelin sheath around axons, essential for the transmission of an action potential. Classically, it has been believed that glia act as the supporting cells of the CNS. In recent years it is becoming clear that this is not their only function.

Neurons are the main signalling units of the CNS. They consist of a cell body, short dendrites, an axon, and synaptic terminals. The dendrites extend out from the neuron, and receive most of the incoming signals from other nerve cells. The tubular axon extends from the cell body, and is the conducting unit for transmitting an action potential to other neurons across a synapse. Neurons can be classified into numerous subdivisions based on their morphology (e.g. unipolar, bipolar), as well as their function, for example; sensory neurons carry information from the periphery into the CNS (perception and motor coordination); motor neurons carry information from the CNS to muscles; interneurons, the largest class, are not specifically sensory or motor, and can be further subdivided into; projection interneurons, which carry signals over a long range within the CNS; and local interneurons, which carry information within

a local area of the CNS. Neurons relay information by releasing neurotransmitter (of which there are numerous types) across a synapse, which dependent on post-synaptic receptors, will be transmitted as an excitatory or inhibitory signal (Kandel et al. 2000).

1:3 Inductive signals control neural cell differentiation

Specification of neural identity within the CNS depends on an interplay between cell intrinsic factors and extrinsic signals. Extrinsic signalling molecules are produced from several defined sources in an around the neural tube. Many of these signals are able to diffuse, allowing them to act over a long range, as well as locally. Exposure of a competent cell to an inductive signal changes the profile of intrinsic molecules expressed by the cell. The competence of a cell to respond to an inductive signal depends on several factors, for example the expression of specific receptors, signal transduction molecules transcription factors that are responsive to an activated signal transduction pathway (Kandel et al 2000). Many transcription factors have been shown to be vital for CNS development, for example *Sox* genes play a role at several stages in neural induction (Collingnon, 1992; Wood and Episkopou, 1999), *Hox* genes in positional information (Tallafuß and Bally-Cuif, 2002), *Pax* and *Nkx* genes are involved in patterning and *Lim* genes in cell fate determination (Lee et al. 2004; Pattyn et al. 2003). In addition, Fibroblast growth factors (FGF) signalling has been shown to be involved in neural induction in the chick (Streit et al. 2000), and in *Xenopus*, Wnt signalling is also involved (Heeg-Truesdell and Labonne, 2006).

In 1924, Spemann and Mangold used amphibian embryos to establish that the differentiation of ectoderm to the neural plate relied on secreted signals from a specialised group of cells that became known as the organiser region (Spemann and

Mangold, 1924). They described the amphibian embryo to be organised in an antero-posterior and dorso-ventral manner. Many groups have tried to identify the precise signals which turn undifferentiated ectodermal cells into precursors of the entire nervous system. Candidates include, noggin (Launay et al. 1996), follistatin and chordin (Sasai et al. 1996; Hemmati-Brivanlou and Melton, 1997; Jessel and Sanes, 2000). These genes all share the ability to inhibit signals transmitted by members of a family of secreted factors, the bone morphogenetic proteins (BMPs) (Jessel and Sanes, 2000). Moreover, *in vitro* studies using cells from *Xenopus* embryos, indicated that undifferentiated ectodermal cells were able to acquire neural markers in the absence of any putative inducers (Sasai et al. 1996). Together these data suggested that ectodermal cells generate neural epithelial cells if ongoing BMP signalling within the ectoderm is inhibited by factors derived from the organizer region (Figure 1.7) (Jessel and Sanes, 2000).

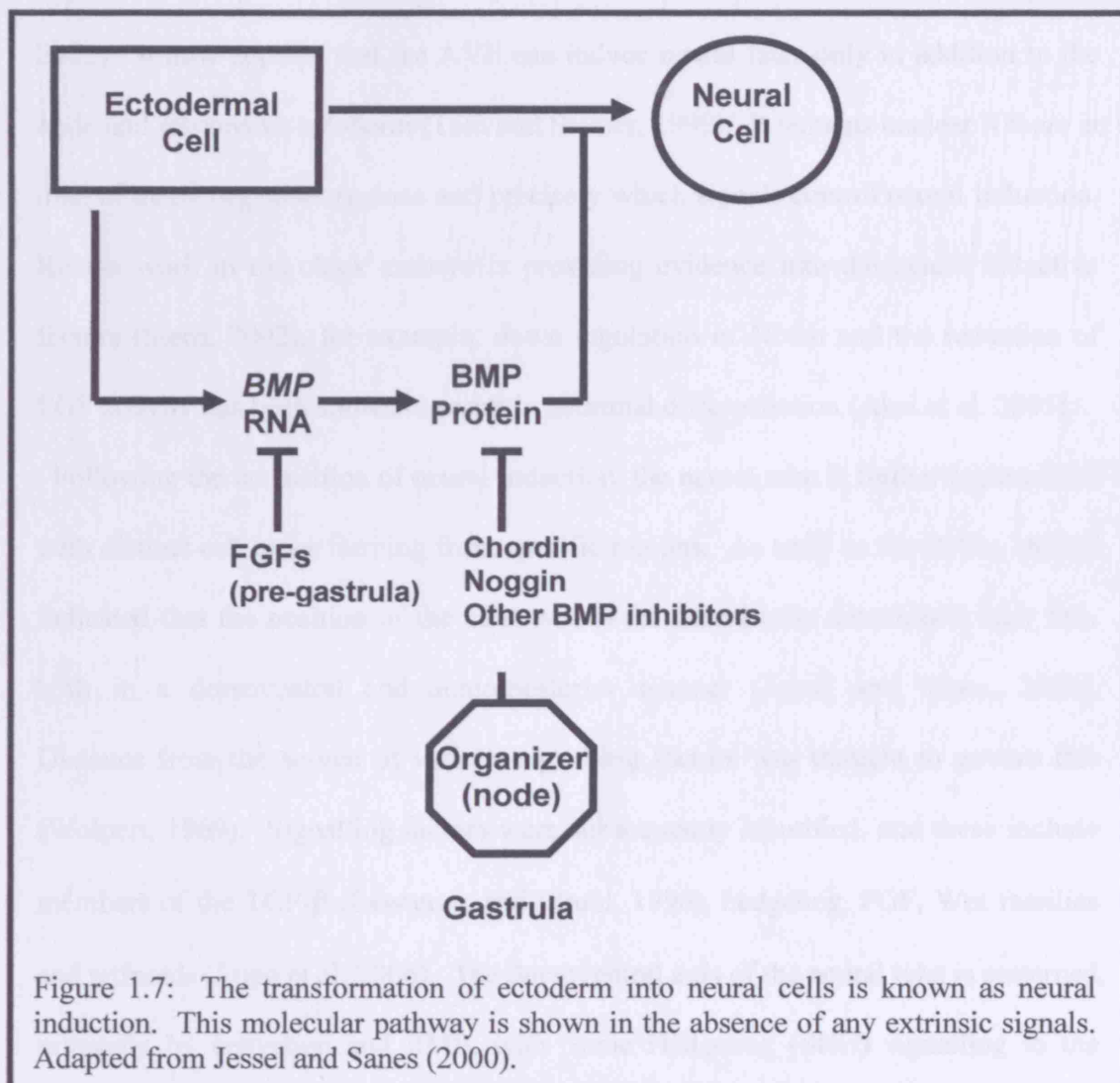


Figure 1.7: The transformation of ectoderm into neural cells is known as neural induction. This molecular pathway is shown in the absence of any extrinsic signals. Adapted from Jessel and Sanes (2000).

However, elimination of the node in mouse (the organizer region) failed to block neural differentiation. This suggests that neural induction is initiated before the formation of the node, in response to signals from other regions of the embryo. Therefore, suppression of BMP signalling may maintain rather than initiate the process of neural induction (Jessel and Sanes, 2000).

It has been suggested that there are separate organiser regions for head, trunk and tail induction (Stern, 2002). The anterior visceral endoderm (AVE) lies outside the organiser region. In the absence of the AVE, the developing forebrain is severely defective (Beddington and Robertson, 1998), therefore was thought to be the 'head'

organiser region. The 'trunk-tail' organiser was believed to be Hensen's node (Stern, 2002). It now appears that the AVE can induce neural fates only in addition to the node and responsive ectoderm (Tam and Steiner, 1999). It remains unclear if there is one, or many organiser regions and precisely which signals control neural induction. Recent work in the chick embryo is providing evidence into the neural inductive factors (Stern, 2002), for example, down regulation of Notch and the reduction of FGF activity has been shown to result in neuronal differentiation (Akai et al. 2005).

Following the acquisition of neural induction, the neural tube is further regionalised with distinct cell types forming from specific regions. As early as the 1940s, studies indicated that the position of the cells within the neural tube determined their fate, both in a dorsoventral and anteroposterior manner (Jessel and Sanes, 2000). Distance from the source of various signalling factors was thought to govern this (Wolpert, 1969). Signalling factors were subsequently identified, and these include members of the TGF- β (Lawrence and Struhl, 1996), hedgehog, FGF, Wnt families and retinoids (Lupo et al. 2006). The dorsoventral axis of the neural tube is patterned, primarily by hedgehog and BMP, with Sonic Hedgehog (SHH) signalling to the ventral neural tube and BMP to the dorsal half (Figure 1.8). The rostrocaudal axis is responsive to retinoids, FGFs, hedgehogs, Wnts and BMPs (Doniach, 1995; Lui et al. 2001) (Figure 1.9). As well as rostrocaudal and dorsoventral signalling systems, local signals between developing neurons are also acting to form all the neuronal subtypes (Lumsden and Krumlauf, 1996).



Figure 1.9: Signalling rostrocaudal patterning in the developing neural tube establishes the major regions of the CNS, the forebrain, midbrain, hindbrain and spinal cord. FGF and Wnt are secreted from the isthmus, and Shh by the node that lies immediately adjacent. Adapted from Jessel and Sanes (2000).

Dorsoventral patterning

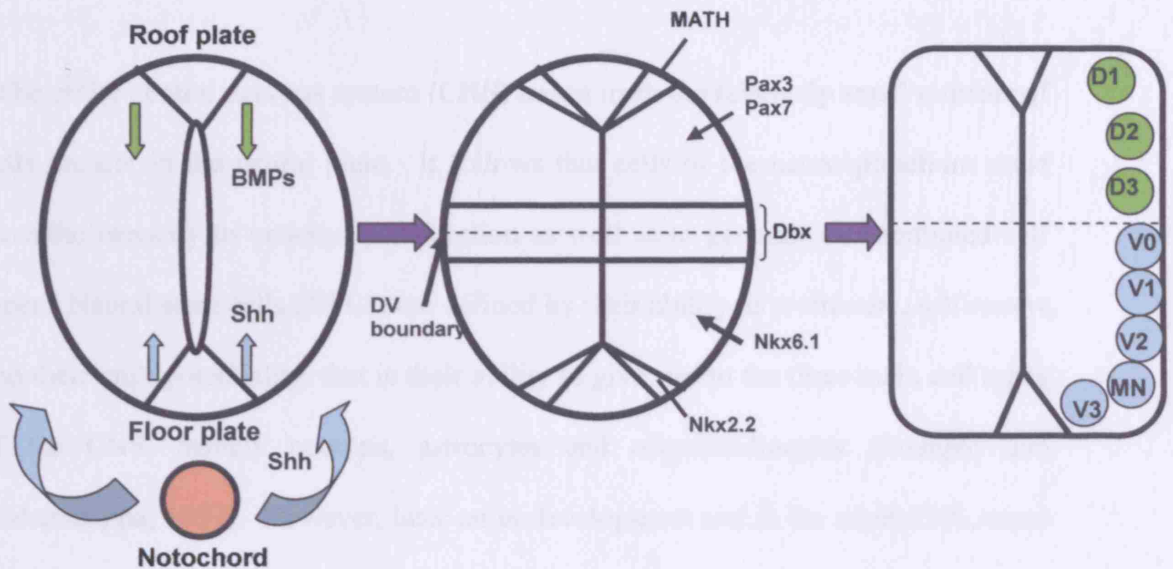


Figure 1.8: Simplified dorsoventral patterning of the ventral neural tube. Sonic hedgehog (Shh) is produced by the notochord and floor plate, and patterns the ventral neural tube. Bone morphogenetic protein (BMP) signalling from the roof plate patterns the dorsal neural tube. Adapted from Jessel and Sanes (2000).

Rostrocaudal patterning

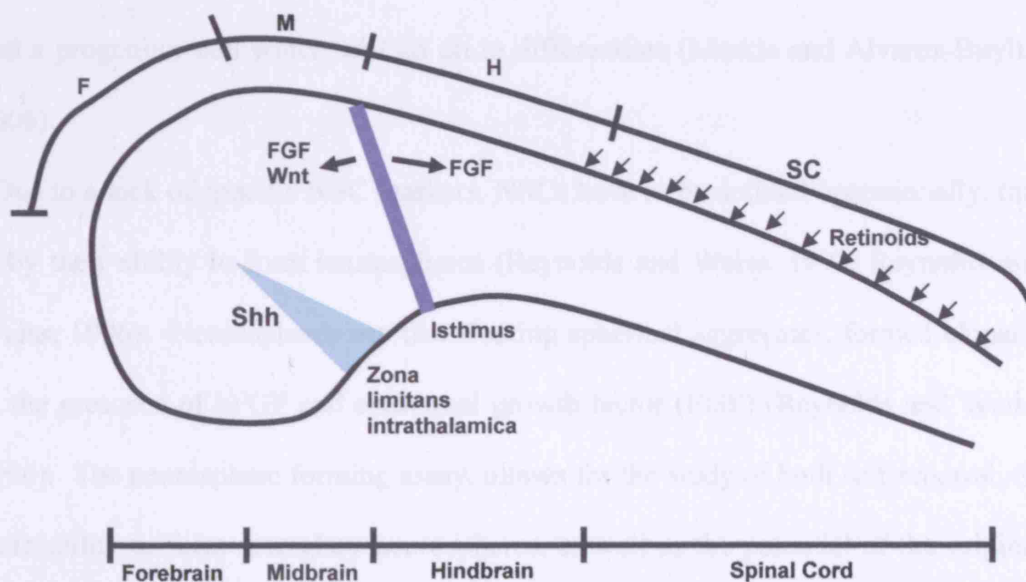


Figure 1.9: Simplified rostrocaudal patterning in the developing neural tube establishes the main subdivisions of the CNS, the forebrain, midbrain, hindbrain and spinal cord. FGF and Wnt are secreted from the isthmus, and Shh by the zona limitans intrathalamica. Adapted from Jessel and Sanes (2000).

1:4 Neural Stem Cells

The entire central nervous system (CNS) arises from the relatively small number of cells present in the neural plate. It follows that cells of the neuroepithelium must have the capacity to undergo proliferation as well as to generate differentiated cell types. Neural stem cells (NSCs) are defined by their ability to proliferate, self-renew, and their multipotentiality, that is their ability to give rise to the three main cell types of the CNS, namely neurons, astrocytes and oligodendrocytes (Stemple and Mahanthappa, 1997). However, later on in development and in the adult CNS, more differentiated cell types, e.g. transit amplifying cells, which have a limited capacity to divide and self-renew (Alexanian and Kurpad, 2005; Morshead et al., 1994) can be mistaken for stem cells. Although, it is apparent that NSCs are retained into adulthood. The multipotent NSC can divide symmetrically, giving rise to 2 NSCs, hence increasing the NSC population, or divide asymmetrically, giving rise to a NSC and a progenitor cell which will go on to differentiate (Merkle and Alvarez-Buylla, 2006).

Due to a lack of specific NSC markers, NSCs have to be defined operationally, that is by their ability to form neurospheres (Reynolds and Weiss, 1992; Reynolds and Weiss, 1996). Neurospheres are free-floating spherical aggregates, formed clonally in the presence of bFGF and epidermal growth factor (EGF) (Reynolds and Weiss, 1996). The neurosphere forming assay, allows for the study of both self-renewal, by their ability to form secondary neurospheres, as well as the potential of the original NSC, by subsequent immunohistochemistry to assay the types of cells differentiating from the neurosphere. The neurosphere assay can also assess NSC potential at specific developmental time points, for example, NSCs isolated from the early CNS only undergoing neurogenesis, and not yet gliogenesis, can provide evidence for

which cues are necessary to enable NSCs to act in a truly multipotent NSC manner. From the embryonic CNS, NSCs can be isolated from all areas including, cerebral cortex, hippocampus, striatum, midbrain (including the substantia nigra), cerebellum and spinal cord (Rajan and Snyder, 2006).

In the mammalian adult, NSCs are believed to reside in the ventricular zone (VZ), a germinal region of the neuroepithelium. Adjacent to the VZ lays a second germinal zone, the subventricular zone (SVZ) and neurogenesis remains ongoing in this SVZ throughout the life of the mammal. Therefore, it is believed to be an adult NSC niche (Tramontin et al. 2003). In the adult, neurogenesis involves the generation of new neurons which mature to replace older neurons under both normal physiological or pathological conditions (Lindsey and Tropepe, 2006). Neurogenesis continues mainly in the olfactory bulb and the hippocampus, but can also be detected in the cortex (Pevny and Rao, 2003). Undifferentiated cells in the SVZ can be identified throughout adulthood, and have been shown to form neurospheres (free-floating spherical structures formed clonally from a NSC, discussed in chapter 3) as well as generate neurons and glia upon differentiation (multipotent). Pevny and Rao (2003) killed actively dividing cells of the SVZ with tritiated-thymidine, and showed that ~1% of the cells in this region were slowly dividing stem cells capable of regenerating the remaining cells in the SVZ.

Mignone et al. (2004) made transgenic mice in which NSCs are marked with a GFP marker gene, under the control of a regulatory region from the gene encoding nestin (an intermediate filament protein). Nestin is believed to be expressed in NSCs both during development and into adulthood. By sorting the GFP positive cells, they found that these GFP nestin positive cells were able to form 70 times more neurospheres than the GFP negative population. The data indicates that GFP/nestin expression is found in areas of active neurogenesis, e.g., the SVZ, rostral migratory

stream (RMS) and dentate gyrus (DG), as well as in the posterior wall of the lateral ventricle, and in the Bergmann glia cells in the cerebellum. In addition to NSCs being discovered in the brain, Weiss et. al. (1996) showed that cells derived from the adult mouse thoracic spinal cord (SC) were able to proliferate to form neurospheres, capable of forming secondary spheres and differentiating into neurons, astrocytes and oligodendrocytes. They also showed that the lumbar/sacral SC and the third and fourth ventricles could give rise to neurospheres. Therefore, they conclude that the SC and entire ventricular neuroaxis of the adult mammalian CNS contains multipotent NSCs, all with proliferation dependent specificity on epidermal growth factor (EGF) and fibroblast growth factor (FGF2). In addition, Alexanian and Kurpad (2005) showed that quiescent neural cells persist in the CNS in the form of dormant progenitors or highly specialised cell types. If exposed to the correct signals, these quiescent cells may be capable of once again acting as NSCs, i.e. able to divide and generate a NSC (self renewal) and differentiated cell types (multipotent). In this experiment, Alexanian and Kurpad (2005) cultured differentiated (quiescent) neural cells with adult NSCs from the hippocampus (bFGF), and found that the colonies forming were similar to NSCs in morphology, proliferation kinetics and marker expression.

1:5 Neural Stem Cells as a Potential Therapy for Neurodegenerative Diseases and Spinal Cord Injuries

Studies with NSCs have raised the hope of their potential to treat neurodegenerative diseases. In the short term, transplantation of NSC may replace damaged or lost cells types. In the long term, better understanding of the signals to which NSC respond, may allow the regulation of adult endogenous NSCs to regenerate lost or damaged CNS tissue. For example, in Parkinson's disease, the symptoms (e.g. tremor and slow movements) do not become apparent until ~80% of dopaminergic neurons of the substantia nigra are already dead (Kandel et al. 2000). Treatments to date only offer short term relief from the symptoms and are by no means a complete cure. The diseased brain is incapable of regenerating the region, therefore therapeutic interventions that replace the dead dopaminergic neurons would provide relief from this disease.

Stem cells in regions other than the CNS have been successfully used in the treatment of many patients over the last 30 years. For example, skin grafts to treat severe burn victims (Gardner, 2007), where stem cells residing in the healthy skin graft, can form healthy new skin in the damaged area. Additionally, bone marrow stem cells are now routinely used to treat patients undergoing therapy for leukaemia (Linker, 2007). However, these are both relatively simple systems compared to the CNS. In the case of spinal cord injuries, there are numerous cell types all with very specific connections (Fernandez et. al., 2006) therefore being able to re-build such an intricate system appears to be a formidable challenge. Some studies have shown promise, for example, either whole neurospheres, or cells derived from neurospheres (Karbanova et. al. 2004; Klein et. al. 2005) have been shown to integrate into a host brain and differentiate to form functioning neurons, astrocytes and oligodendrocytes.

1:6 Aims of this thesis

The objective was to understand the factors and signals that regulate NSCs in the CNS. First, I wished to characterise the earliest time at which the mouse CNS can form neurospheres, as well as establishing neurosphere forming conditions and time points for the chick CNS. Secondly, I focussed on the expression of the transcription factor SOX9 at time points before and after neurosphere formation, as well as in the adult. The requirement for *Sox9* in the generation of neurospheres was then assessed. Thirdly, I assayed the role of Sonic hedgehog (SHH) in NSC proliferation, and neurosphere forming ability at different developmental stages, both in the mouse and chick. Finally, the relationship between *Sox9* and SHH in neurosphere formation was examined.

Chapter 2

Materials and Methods

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2.1 Mouse Lines

2.1.1 Animal care

Animals were kept on a 12 hour light-dark cycle, and food and water were provided at all times. The day of detection of a vaginal plug was taken as 0.5 days *post coitum*, assuming the time of conception was half way through the dark phase. All methods were carried out in accordance with the Animals (in Scientific Procedures) Act 1986.

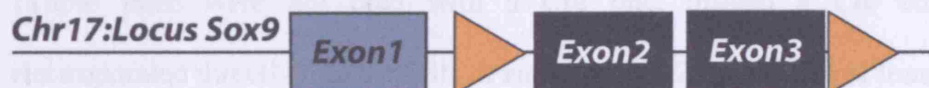
2.1.2 Genotyping

Tail pieces or ear pieces were lysed overnight at 55°C in tail lysis buffer (see appendix) with 100µg/ml proteinase K. Phenol extraction was performed by adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). Samples were then centrifuged using a desktop centrifuge (Heraeus) at 15,000g for 15 minutes. The top, aqueous phase was used for PCR.

2.1.3 Flox-Sox9

Flox-Sox9 mice were obtained from Andreas Schedl (Chaboisser et al. 2004). The orange arrows in the schematic below indicate the loxP sites, upon cre recombination, Exon2 and Exon3 will be excised.

Flox-Sox9:



To generate *Sox9* null embryos, Flox-*Sox9* heterozygous females were bred with Zone pellucida 3 (Zp3)-Cre (oocyte specific) males to generate Zp3-Cre/+; *Sox9*^{flox/wt} females. Flox-*Sox9* heterozygous males were bred with Protamine (Prm1)-Cre (spermatic specific) females to generate Prm1-Cre/+; *Sox9*^{flox/wt} males. The *Sox9* null embryos were then generated by crossing Zp3-Cre/+; *Sox9*^{flox/wt} females, with Prm1-Cre/+; *Sox9*^{flox/wt} males.

The Flox-*Sox9* embryos were genotyped as described in Chaboissier et al. (2004). Wild type and *Sox9*^{flox} alleles were identified using the primers 5'-GGGGCTTGTCTCCTTCAGAG-3', or 5'-ACACAGCATAGGCTACCTG-3' and 5'-TGGTAATGAGTCATACACAGTAC-3', respectively. The *Sox9* null allele was identified using the primers 5'-GTCAAGCGACCCATG-3' and 5'-TGGTAATGAGTCATACACAGTAC-3'.

2.1.4 Z/*Sox9*

Z/*Sox9* mice were obtained from Kathy Cheah (unpublished data). The floxed allele can be genotyped by the presence of LacZ upon visualisation (see appendix for lacZ staining solution). Upon Cre recombination, *Sox9* will be miss-expressed driven by a CMV chicken β -actin promoter, and GFP (green fluorescent protein) visualised.

Z/*Sox9*:



These mice were not bred with a Cre line, instead a Cre construct was electroporated directly into the cells *in vitro* (see 2.3.2 for method of transfection).

2.1.5 Xt^J

The extra toes (Xt)^J mutation is a mutation of the *Gli3* allele, possessing a deletion of the 3' sequence (Hui and Joyner, 1993). Heterozygous mice exhibit preaxial polydactyly, and homozygous mice exhibit craniofacial abnormalities, exencephaly polydactyly and die around birth.

The presence of the wild type *Gli3* allele was detected using the primers:

5'-GTTGGCTGCTGCATGAAGACTGAC-3',

5'-GGCCCAAACATCTACCAACACATAG-3'

The presence of the mutant allele was detected with the primers:

5'-TACCCCAGCAGGAGACTCAGATTAG-3',

5'-AAACCCGTGGCTCAGGACAAG-3'.

2.2 The Neurosphere Assay

2.2.1 Dissection of the dorsal telencephalon

Pregnant female mice (MF1 wild type mice were used in most experiments unless otherwise stated) were killed at the required day of gestation (from E9.5 to E14.5) by cervical dislocation (schedule 1 method). The uterine horns were removed, and the embryos dissected free from their deciduas in PBS-A. Heads were removed from each embryo and transferred directly into DMEM-F12 media containing 100U/ml penicillin and 100µg/ml streptomycin sulphate (both Gibco) on ice until further dissection. (If MF1 mice were not used, and genotyping was required, embryos were kept separate). Under a dissecting microscope, watchmakers forceps were used to peel away the skin and skull (at later stages only) overlying the telencephalon. The tissue surrounding both lobes of the telencephalon was then removed, and the two lobes were separated. Remaining membranes were peeled off from both lobes, and

the ventral telencephalon removed. The two lobes were then placed in fresh, ice cold DMEM-F12 (containing penicillin and streptomycin sulphate) swabbed with 70% ethanol and transferred to a tissue culture class II cabinet.

2.2.2 Dissection of the Spinal Cord

Pregnant female mice (MF1 wild type mice were used in most experiments unless otherwise stated) were killed at the required day of gestation (from E9.5 to E14.5) by cervical dislocation (schedule 1 method). The uterine horns were removed, and the embryos dissected free from their deciduas in PBS-A. Heads were removed from each embryo and the bodies transferred directly into DMEM-F12 media containing 100U/ml penicillin and 100µg/ml streptomycin sulphate (both Gibco) on ice until further dissection. (If MF1 mice were not used, and genotyping was required, embryos were kept separate). Under a dissecting microscope, watchmakers forceps were used to peel away the skin (at later stages only) and underlying membranes. At later stages, the cartilage also needs to be removed. The full length of the spinal cord was then dissected by removing the surrounding tissue. The spinal cords were then placed in fresh, ice cold DMEM-F12 (containing penicillin and streptomycin sulphate) swabbed with 70% ethanol and transferred to a tissue culture class II cabinet.

2.2.3 Generation of Neurospheres

The tissue (either dorsal telencephalon lobes, or spinal cords) were transferred to a universal tube, and 0.5ml neurosphere proliferation medium (NPM) added. First a P1000 Gilson (filter tip) was used to roughly dissociate the tissue, then a plugged fire-polished Pasteur pipette (50-100 µm tip diameter) was used to finely dissociate the tissue to a single cell suspension. The single cell suspension was then made up to

1ml with NPM, and passed through a 40µm cell strainer (Falcon). At this point the number of cells was calculated using a haemocytometer, and cells were then plated at a density of 5×10^4 cells/ml in NPM in the appropriate tissue culture dish (2-3ml in a 3.5cm dish, 5-6ml in a 6cm dish, 8-10ml in a 10cm plate). The cells were then cultured at 37°C, 5% CO₂. 24 hours later, cells were dissociated a second time, and passed through a cell strainer again if necessary, and 1-2ml NPM added. A further 48 hours later, a full medium change was carried out either by centrifugation (1000rpm for 5 minutes) or by gravity, then aspirating the NPM and replaced with fresh NPM. The primary neurospheres formed in this assay were kept in culture for a total of 10 days, with a medium change every day, after this point the number of primary neurospheres was counted.

2.2.4 Generation of Neurospheres from the adult Subventricular Zone

Six MF1 8 week old females were killed by cervical dislocation (a schedule 1 method). The head and neck area of each mouse was swabbed with 70% ethanol, and the head removed. The skin was peeled back, and the skull removed to reveal the brain, the whole brain was then removed and placed on the lid of a tissue culture dish. Using a sterile razor blade, a slice of brain was cut in the rostral area to reveal the subventricular zone (SVZ). Using watchmakers forceps, the SVZ was then dissected out, and placed in ice cold DMEM-F12 (containing penicillin and streptomycin sulphate). The plate was then swabbed with 70% ethanol and transferred to a class II tissue culture cabinet, and placed in a universal tube. The tissue was then centrifuged (1000rpm for 5 minutes), and the supernatant aspirated. 4.5ml DMEM-F12 was combined with 500µl papain (final concentration of 1mg/µl) and 1.5µl DNase, 200µl of this solution was added to the tissue, and using a P200 Gilson, the tissue was roughly dissociated. The remaining Papain solution was then

added to the tissue and placed in an incubator (37°C, 5% CO₂) for 30-45 minutes to dissociate further. Following this, the suspension was centrifuged again (1000rpm, 5 minutes), and the supernatant removed and replaced with 1ml NPM, the cell suspension was passed through a 40µm cell strainer (Falcon). The number of cells was then calculated using a haemocytometer, and made up with an appropriate volume of fresh NPM, and plated at a density of 5×10^4 cells/ml, in a tissue culture dish. The cells were then incubated at 37°C, 5% CO₂. The primary neurospheres were cultured for 10 days, with a medium change every 2 days (cells/neurospheres were centrifuged at 1000rpm, 5 minutes, and supernatant removed and replaced with fresh NPM).

2.2.5 Generation of Secondary Neurospheres

After 10 days in culture, primary neurospheres were centrifuged (1000rpm, 5 minutes), the supernatant removed and replaced with 1ml fresh NPM. Using first a P1000 Gilson then a fire-polished Pasteur pipette, the neurospheres are dissociated to a single cell suspension, then passed through a 40µm cell strainer (Falcon). The number of cells was calculated using a haemocytometer, then the cell suspension made up to an appropriate amount with fresh NPM and plated at a density of 5×10^4 cells/ml in a tissue culture dish (Corning). Medium was changed every day for embryonic tissue, and every second day for adult tissue in the same manner as described above. Secondary neurospheres were kept in culture for 10 days at 37°C, 5% CO₂.

2.2.6 Differentiation of Neurospheres

10 day old primary or secondary neurospheres were placed in 0.5ml of neurosphere differentiation medium (NDM) on Matrigel coated Sonic Seal (Lab-Tek) plates (Matrigel was diluted 1:10 with NDM, and pipetted into each well of the Sonic Seal plates, enough to cover the bottom of the plates, then incubated at 37°C, 5% CO₂ for 30-60 minutes). Neurospheres were allowed to differentiate for 5 days at 37°C, 5% CO₂, with a medium change every day with fresh NDM.

2.2.7 Generation of Neurospheres from Chick CNS tissue

Fertilized chicken eggs were obtained from the Winter Egg Farm, and incubated at 37.5°C for 3-6 days. At the stage required, chicks were removed from their shells, and placed ice cold DMEM-F12 (containing penicillin and streptomycin sulphate) until further dissection. The spinal cord and dorsal telencephalon were then dissected in PBS-A, and the dissected tissue placed back into ice cold DMEM-F12 (containing penicillin and streptomycin sulphate). The plate containing the tissue was swabbed with 70% ethanol and transferred to a class II tissue culture cabinet. From this point onwards the methods of generating neurospheres, forming secondary spheres and differentiation was the same as described above.

2.3 Treatment of Neurospheres

2.3.1 Addition of SHH or Cyclopamine to Neurospheres

2.3.1.a Mouse

Three days after originally plating the cells, SHH, the control protein (CP)* or Cyclopamine (200µM) was added directly into the culture medium (after a full medium change). Neurospheres / cells were then cultured as described above. After

10 days total in culture, each condition is assessed for neurosphere forming ability, if any. Each experiment is repeated 12 times, unless otherwise stated.

2.3.1.b Chick

SHH, CP* or cyclopamine (200 μ M) were added to the culture medium one day after plating the cells in culture. Then neurospheres were then cultures as described above.

SHH protein was collected from cells infected with a Shh virus, and concentrated down 50 times. * The control protein, was collected from the same cell which had not been infected with Shh virus, and concentrated down 50 times.

2.3.2 Nucleofection

Single cells from either the dorsal telencephalon or spinal cord of mouse embryo (E9.5 and E10.5), or SVZ cells, were allowed to recover for 24 hours in culture. After this all clumps which might have formed were dissociated to single cells, centrifuged (1000rpm, 5 minutes) and the supernatant aspirated. Cells were resuspended in 100 μ l Amaxa nucleofection solution (following the Amaxa, electroporation of mouse neural stem cells protocol), and 5 μ g of DNA added to the solution. This cell suspension was transferred to an Amaxa cuvette and then to the nucleofector devise (Amaxa), and once again following the Amaxa manual for electroporation of mouse neural stem cells, cells were nucleofected. Cells were then immediately transferred to pre-warmed and gassed medium, and incubated at 37°C, 5% CO₂. All constructs used were tagged with GFP, so 24 hours after nucleofection, cultures were checked for the presence of GFP to check for successful transfection.

For each electroporation, an equal number of cells were electroporated with GFP alone as a control. For each construct, the transfection is repeated 8 times.

2.3.3 *In ovo* Electroporation

Fertilized chicken eggs were obtained from the Winter Egg Farm, and incubated at 37.5°C for 3 days. 3ml of albumen was removed from each egg with a syringe, after which a window was cut in the shell. Using a pulled glass needle, 1-2µg/µl of the plasmid DNA in TE pH8.0 with bromophenolblue was injected into the neural tube with an injectmatic picopump. A drop of PBS-A was placed either side of the neural tube and electrodes (also dipped in PBS-A) were positioned either side of the neural tube and electroporated with six 50msecond 40V pulses, using a BTX ECM-830 electroporator. The window in each shell was then sealed with tape, and the eggs placed back in the incubator at 37.5°C for 24 hours. After this time, surviving chicks were removed from each egg and placed into ice cold PBS-A, each in a separate well, and the presence of GFP was assessed for each embryo. Those that express GFP (electroporation had been successful) were then dissected for their spinal cords, after which the neurosphere forming assay is carried out as previously described. For each construct, 8 chick neural tubes are electroporated, and the spinal cords from all are pooled together. This is then repeated 4 separate times unless otherwise stated.

2.4 Immunohistochemistry

2.4.1 Neurospheres

10 day old primary or secondary neurospheres were allowed to pellet by gravity in a microcentrifuge tube, and the supernatant removed. Neurospheres were rinsed briefly with PBS-T (PBS with 0.1% Triton X-100), and fixed on ice with 4%

paraformaldehyde (in PBS) for 20 minutes. They were then rinsed 3 times 5 minutes with PBS-T (each time allowing the spheres to pellet by gravity, and aspirating the supernatant). Following this PBS-T with 1% sheep serum was incubated with the neurospheres for 30 minutes. Primary antibodies were diluted to the required dilution with PBS-T (1% sheep serum), and incubated with the neurospheres overnight at 4°C. The following day, neurospheres were rinsed 3 times with PBS-T, each time for 5 minutes, then spheres were incubated with specific secondary antibodies (either Alexa 488 or Alexa 594 conjugated antibodies) diluted 1:350 with PBS-T (1% sheep serum) for 1.5 hours at room temperature in the dark. Samples were subsequently rinsed 3 times with PBS-T (each for 5 minutes), and mounted in Vectashield with 4',6-Diamidino-2-phenylindole (DAPI) (Vector Laboratories) on Superfrost slides. All immunofluorescence images were obtained with a Leica TCS SP confocal using a UV 10x/0.4 or 40x/0.5 NA dry HC-PLAPO lens (Leica). Images were processed using the ImageJ 1.30v and Adobe Photoshop CS v8.0 (Adobe Systems) software packages.

Differentiated neurospheres were treated in the same manner, with the only difference being, the neurospheres are attached rather than floating, therefore, supernatant can be directly removed from each well, rather than waiting for them to pellet.

2.4.2 Preparation of embryos for cryosectioning and immunohistochemistry

Mouse and chick embryos were fixed in 4% paraformaldehyde in 0.1M phosphate buffer (PB) for 1-3 hours at 4°C (depending on the stage of the embryo). Embryos were then rinsed in PB 3 times, and allowed to sink in 30% sucrose solution. Embryos were then positioned in OCT in Dispomoulds and frozen (using dry ice) in place. Frozen samples were stored at -80°C until ready for sectioning. 12µm

sections were cut with a Cryostat (Leica), and transferred onto Superfrost plus slides (BDH). Slides were stored at -80°C until required.

Slides were removed from -80°C freezer, and allowed to defrost at room temperature. A line was then drawn with a wax pen (DAKO) around the sample, and this was allowed to dry. OCT was then washed off with PBS-A, and blocked with PBS-T with 1% sheep serum for 30 minutes. Sections were then incubated with a primary antibody diluted with PBS-T with 1% sheep serum overnight in a humidified chamber at 4°C. The next day, sections were rinsed with PBS-T three times, 5 minutes each, then incubated with the appropriate secondary antibody, diluted in PBS-T, 1% sheep serum for 1.5 hours, in a dark humidified chamber at room temperature. Slides were subsequently rinsed three times with PBS and mounted with Vectashield with DAPI (Vectashield laboratories), and visualised as previously described.

2.4.3 Antibodies

Primary Antibodies

| Primary Antibody | Dilution | Antigen Recognized | Cell Type Recognized |
|---|----------|---|----------------------|
| Sox2 Rabbit Polyclonal Chemicon | 1:1000 | SOX2 | |
| Sox9 Rabbit Polyclonal Gift from Silvana Guioli | 1:1000 | SOX9 | |
| Sox10 Rabbit Polyclonal Gift from Martin Cheung | 1:1000 | SOX10 | |
| GFAP Mouse Monoclonal Cy3 conjugate IgG Sigma | 1:200 | Glial Fibrillary Acid | Astrocytes |
| TuJ1 Mouse Monoclonal IgG Covance | 1:1000 | β -III Tubulin | Neurons |
| CNPase Chemicon | 1:100 | 2',3'-cyclic nucleotide-3'- phosphodiesterase | Oligodendrocytes |
| O4 (clone 81) Mouse monoclonal Chemicon | 1:1000 | Oligodendrocyte marker | Oligodendrocytes |

Secondary Antibodies

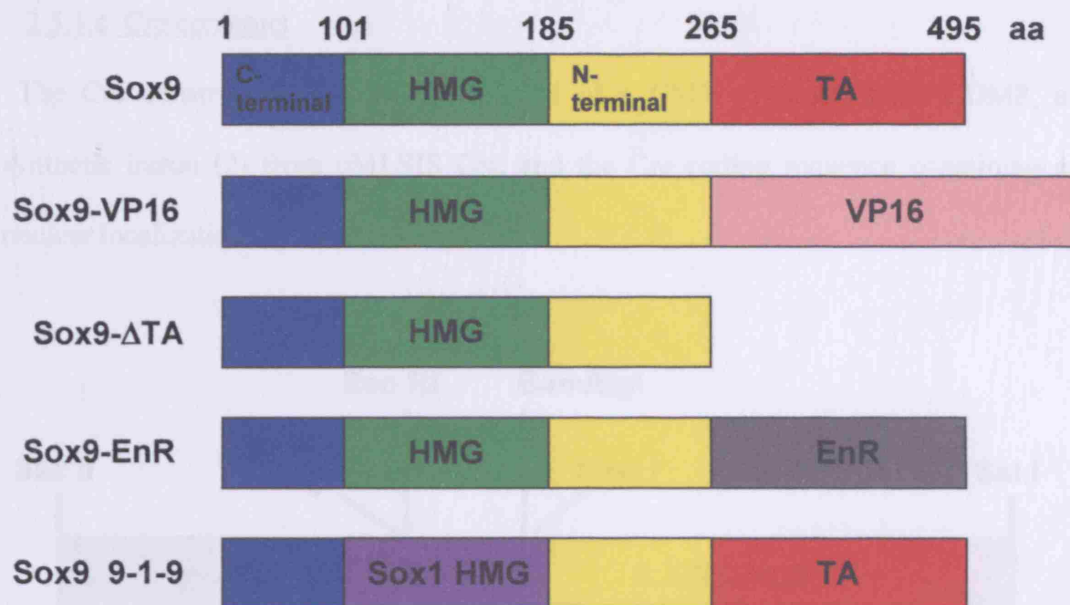
| Secondary Antibody | Dilution | Primary Antibody Recognized |
|--------------------------------|----------|-----------------------------|
| Goat Anti-Mouse Alexa 594 IgG | 1:350 | Mouse Monoclonal |
| Goat Anti-Mouse Alexa 488 IgG | 1:350 | Mouse Monoclonal |
| Goat Anti-Mouse Alexa 488 IgM | 1:350 | Mouse Monoclonal |
| Goat Anti-Rabbit Alexa 594 IgG | 1:350 | Rabbit Polyclonal |
| Goat Anti-Rabbit Alexa 488 IgG | 1:350 | Rabbit Polyclonal |

2.5 Constructs

2.5.1 Constructs used

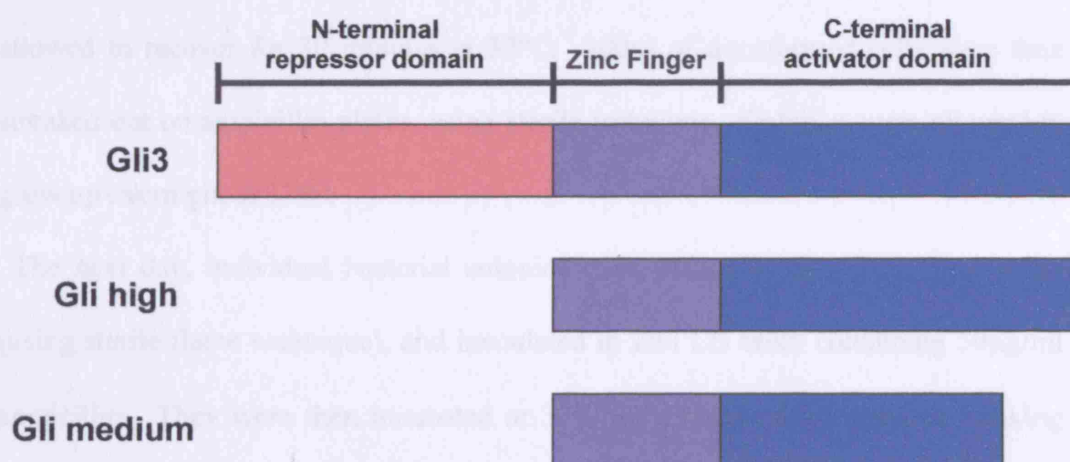
2.5.1.a Sox9 constructs

Chick *Sox9* cDNA (Kamachi et al. 1999) was inserted upstream of an internal ribosomal entry site (IRES) and a nuclear localization sequence (nls) tagged GFP in pCAGGS expression vector (Niwa et al. 1991; Cheung and Briscoe, 2003). pCAGGS-IRES-nls-GFP (Cheung and Briscoe, 2003) was used as a negative control in all electroporation experiments. Sox9-VP16, the transactivation domain was replaced with the heterologous VP16 transactivator domain (Cheung et al. 2005) Sox9- Δ TA acted as a dominant negative *Sox9* construct, and was made by the removal of the transactivation (TA) domain (Cheung et al. 2005), both constructs were ampicillin resistant. Sox9EnR also acted as a dominant negative *Sox9* construct, the C-terminal transactivation region was replaced with the engrailed repressor domain (EnR) (Cheung et al. 2005). Sox9 9-1-9, was full length *Sox9* with the Sox9 HMG domain removed and replaced with Sox1 HMG domain (Kamachi et al. 1999). S64A S181A (Gift from Martin Cheung) is a double alanine mutation at two phosphorylation sites of *Sox9*.



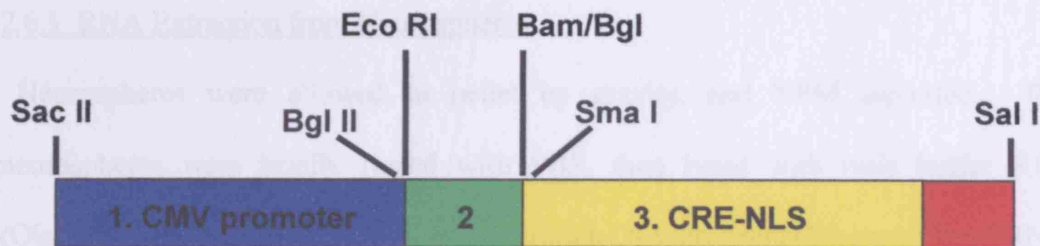
2.5.1.b Gli constructs

Gli3 activator constructs were designed using human *GLI3*, and cloned into the expression vector pCAGGS (Niwa et al. 1991) upstream of an IRES and a nls tagged GFP (Stamatakis et al. 2005). Gli3 activator function was revealed by the removal of the N-terminal repressor domain, both constructs were ampicillin resistant. A dominant negative form of Gli consisted of the Zinc Finger (ZnF) domain of Gli3 alone, therefore blocking both activator and repressor function (Cayuso et al. 2006).



2.5.1.c Cre construct

The Cre construct (O’Gorman) consisted of a CMV promoter from CDM8, a synthetic intron (2) from pMLSIS Cat, and the Cre coding sequence containing a nuclear localization signal (3).



2.5.2 Transformation of DH5 α cells and Midi prep

Luria-Bertani (LB) agar plates containing ampicillin (100 μ g/ml) were warmed in a 37°C oven. DH5 α *E.coli* competent cells (Stratagene), stored at -80°C were thawed on ice. 2 μ g plasmid DNA was mixed with 200 μ l DH5 α cells for 30 minutes on ice, cells were then incubated at 42°C (dry heat block) for 90 seconds and immediately placed on ice for 2 minutes. LB broth was added to the cells, which were then allowed to recover for 30 minutes at 37°C. 200 μ l of transformed cells were then streaked out on ampicillin plates, using sterile technique. Colonies were allowed to grow up overnight at 37°C.

The next day, individual bacterial colonies were picked using a P200 pipette tip (using sterile flame technique), and inoculated in 2ml LB broth containing 50 μ g/ml ampicillin. They were then incubated at 37°C in a Brunswick Scientific Shaking Incubator for 8 hours at 200rpm. This culture was then expanded in 90ml LB broth containing 50 μ g/ml ampicillin, and incubated at 37°C in a Brunswick Scientific

Shaking Incubator for 16 hours at 250rpm. The DNA was then extracted from the DH5 α cells using a Qiagen midiprep kit.

2.6 RNA extraction and RT-PCR

2.6.1 RNA Extraction from Neurospheres

Neurospheres were allowed to pellet by gravity, and NPM aspirated. The neurospheres were briefly rinsed with PBS, then lysed with lysis buffer RLT (Qiagen), then homogenized by passing through QIAshredders (Qiagen). Total RNA was extracted from neurospheres following the Qiagen RNAeasy Midi protocol for animal cells (Qiagen). RNA was eluted in nuclease-free water. The RNA was then quantified using a NanoDrop ND-1000 spectrophotometer (Labtek).

2.6.2 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA samples were treated with DNase I (Promega) for 30 minutes at 37°C and reverse transcribed for 1 hour at 37°C using Omniscript RT (Qiagen) with Random Decamers (Ambion). 2 μ g of starting cDNA was used for each PCR reaction employing gene-specific primers. The PCR profile consisted of 94°C for 15 minutes (1 cycle), 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds (30 cycles) and 72°C for 8 minutes (1 cycle), followed by a hold at 10°C. A Peltier Thermal Cycler (MJ Research) was used. β -actin primers were used as a positive cDNA control.

2.6.3 RT-PCR Primer Sequences

| Primers | Sequence |
|----------------|--|
| Sox2 | 5'-CACAACTCGGAGATCAGCAA-3' 5'-CTCCGGGAAGCGTGTACTTA-3' |
| Sox8 | 5'-AGGCGAAGGAAGAGTGTGAA-3' 5'-CCCTTCCAGCCTTAGCTCTT-3' |
| Sox9 | 5'-AGCTCACCAGACCCTGAGAA-3' 5'-CTCCAGCAATCGTTACCTTC-3' |
| Sox10 | 5'-AGGCCTCACTGCTCCTGTTA-3' 5'-TTGACCAGTTCCCACATTCA-3' |
| β -actin | 5'-TCATGCCATCCTGCGTCTGGACCT-3' 5'-CCGGACTCATCGTACTCCTGCTTG-3' |

Chapter 3

Timing of Neurosphere Formation in the Mouse, and Establishing Neurosphere Formation in the Chick

Chapter 3

Timing of neurosphere formation in the mouse, and establishing neurosphere formation in the chick

3:1 Introduction into neurospheres as a way to assay NSC behaviour

Little is known about the signals neural stem cells (NSCs) respond to, in part because of the difficulty of identifying NSCs prospectively. Due to the lack of specific NSC markers, they have to be defined operationally, by their ability to self-renew and maintain multipotentiality through successive passages. The most frequently used assay for this is the ability of a NSC to form a neurosphere. Neurospheres are floating spherical structures, composed of a heterogeneous population of cells including NSCs, neural progenitor cells (NPC) as well as differentiated cell types (Campos, 2004). They can be derived from a single NSC. Reynolds and Weiss (1992) characterised the neurosphere forming assay from the embryonic and adult mouse brain. They described a serum-free method of propagating spheres from a small population of NSC/ NPC in the presence of epidermal growth factor (EGF). These neurospheres could form at various plating densities and possessed the ability to form numerous secondary neurospheres (self-renewal) and to generate the three major cell types of the CNS, namely, neurons, astrocytes and oligodendrocytes, upon differentiation (multipotent). The cells initiating neurosphere formation therefore had the characteristics of NSCs. In the years to follow it was shown that a consistent, renewable source of neurosphere forming cells could be grown in the presence of both EGF and basic fibroblast growth factor (bFGF) (Reynolds and Weiss, 1996). It was subsequently noted that

the bFGF-responsive NSC and EGF-responsive NSC are two separate populations of cells each with separate proliferative kinetics during development (Martens et al., 2000). FGF-responsive stem cells can be found from E8.5 (mouse), and the EGF-responsive stem cells are believed to arise from the FGF-responsive cells between E11 and E13 (Tropepe et al., 1999).

The neurosphere formation assay presents a highly useful tool to assay both multipotentiality, by differentiation studies, and self renewal, by their ability to clonally form secondary neurospheres upon dissociation of the primary neurospheres. Some studies have used the neurosphere assay to retrospectively identify NSCs, both in the embryo and the adult (Engstrom et al., 2002). However, the survival rate of an isolated cell is low, probably reflecting the difference from the *in vivo* environment where a NSC would be surrounded by many other cell types. For this reason numerous groups calculate a plating density for their specific cell type predicted to produce clonal neurosphere formation. In practice, however, the neurospheres will often arise from 2 or more NSCs (Andoniadou, unpublished data).

Bez et al. (2003), performed a cross comparison of neurospheres derived from six human cell lines, which were obtained from 12 week old human embryonic CNS. Within one culture (all the cells within the culture were treated in an identical manner) they observed different sizes of neurospheres. The larger neurospheres had a darker core, and smaller spheres appeared more translucent. Ultrastructural analysis of these neurospheres showed that phagocytosis of apoptotic bodies occur predominantly within the inner regions of the sphere, and further provide evidence for the presence of lysosomes. High transcriptional activity was discovered in the peripheral cells (evaluated by RNA synthesis). Bez et al. (2003) also showed that cells derived from a dissociated neurosphere were not synchronised, and represented all phases of the cell cycle, the majority of cells over a 10 day period were in G0/G1

phase of the cell cycle. Furthermore, they found that more than 80% of the cells from a dissociated sphere were viable, in the presence of bFGF and EGF.

The *in vitro* assay for neurosphere formation to study NSC behaviour has come under some criticism. Firstly, neurospheres do not occur within the CNS and therefore cannot provide a perfect representation of NSC behaviour *in vivo*. Secondly, there is some doubt as to how long the neurospheres have to be kept in culture undergoing successive passages, to truly show self-renewal capability. Reynolds and Rietze (2005) state that in some cases neurospheres could only be maintained in culture for 3 passages, they conclude that these neurospheres have been generated by neural progenitor cells (NPCs) and not true NSCs. They also find that these particular neurospheres were not able to give rise to all three neural cell types. From these findings they suggest that neurosphere number cannot be directly linked on a one to one basis to NSC number. Moreover, for a definitive identification of a NSC, at least five passages should be performed and multipotentiality maintained throughout this time.

Despite this criticism, many groups continue to use neurosphere cultures as a method to obtain NSCs and study their behaviour. Karbanova et al. (2004), isolated neural cells from E14 rat forebrains and cultured them in the presence of both EGF and bFGF to form neurospheres. They grafted whole neurospheres into intact rat brains, and after 3 weeks examined the graft by immunohistochemistry. They confirmed that after transplantation these neurospheres were able to give rise to neuronal and glial cells, therefore concluding that neurospheres do contain cells able to fulfil the NSC criteria and can successfully integrate into the host brain.

In addition, neurospheres have been shown to maintain some regional specification, even after expansion *in vitro* (Parmar et al., 2002; Klein et al., 2005). Parmar et al. (2002) dissociated neural cells from distinct regions of the embryonic mouse

telencephalon, and cultured them in the presence of either bFGF or EGF or both, to assay neurosphere formation and potential. The subsequent neurospheres from distinct regions of the telencephalon, possessed different responses to FGF and EGF, but cells from all regions proliferated the most in the presence of both bFGF and EGF. Providing evidence that neural cells from different regions of CNS, do maintain regional character *in vitro*. Parmar et al. (2002) showed that many of the genes expressed in proliferating neurospheres were the same as those expressed during development *in vivo*. In addition, upon differentiation of the neurospheres, many of the developing neurons expressed transcription factors known to be regionally expressed in the developing neurons *in vivo*. Klein et al. (2005), first describe a stem cell population residing in the embryonic and adult cerebellum. They show that embryonic and adult cerebellar stem cells (expanded as neurospheres in the presence of bFGF and EGF) are able to give rise to neurons that resemble select subclasses of the *in vivo* cerebellum. Upon transplantation, these neurons were able to acquire positional and mature electrophysiological properties of cerebellar granule cells. Moreover, neurospheres derived from the forebrain and cerebellum gave rise to progeny in accordance with their region of origin. Forebrain derived NSCs, could not adopt cerebellar neuronal character upon transplantation into the neonatal cerebellum. In contrast to these two studies, Santa-Olalla et al. (2003) report that neurospheres derived in the presence of EGF, from the embryonic telencephalon, mesencephalon and rhombencephalon do not maintain regional characteristics. Neurospheres from each of these areas did not express their appropriate antero-posterior (AP) or dorso-ventral (DV) specific markers. They concluded that positional identity of NSCs *in vivo* is due to external signalling factors and not their intrinsic identity. One key difference between these studies is that Santa-Olalla et al. (2003) expanded their neurosphere cultures in the presence of

EGF alone, whereas Parmar et al. (2002) and Klein et al. (2005) cultured their neurospheres in both bFGF and EGF. It may be possible that only bFGF-responsive NSCs, or cells responsive to both EGF and bFGF, but not EGF alone, are able to maintain regional characteristics. Further evidence for the regionalisation of gene expression in NSCs, stems from a study using a transgenic mouse line, in which a *Sox2* (expressed in the early developing CNS) regulatory sequence directs the expression of a β -geo transgene. This regulatory region is required to drive the expression of the β -geo transgene in the developing dorsal telencephalon, and this expression can be maintained *in vitro* over long periods of time in the presence of bFGF and EGF. In comparison the transgene was not expressed in the spinal cord, therefore showing regional specificity maintained *in vitro*.

To date, the neurosphere forming assay remains the best available method to study NSC behaviour. Neurospheres possess the necessary characteristics used to define NSCs, namely, the ability to form clonally, proliferate, self-renew and generate all three major cell types of the CNS (multipotentiality). However, the embryonic ages at which neurospheres can form have not been analysed. In this chapter, I aim to determine the earliest embryonic time points that neurospheres can be generated both from the embryonic mouse dorsal telencephalon and spinal cord. Defining the precise developmental stage at which neurospheres can be derived, will augment the ability to discover factors essential for NSC proliferation and multipotentiality. To date whether the chick CNS tissue can generate neurospheres has not been determined. Therefore, the second aim of this chapter was to establish the embryonic chick CNS as a novel model to assay NSC forming neurospheres, define these as bonafide neurospheres, as well as establishing the developmental time points at which neurospheres can form in this species. Apart from evolutionary comparisons, the use of the chick in addition to the mouse will allow the utilization of the well

defined experimental technique of *in ovo* electroporation, which can in turn provide further understanding of the factors essential for neurosphere formation from NSCs.

3:2 Results

3:2:1 Establishing the time point neurospheres can form from the embryonic mouse CNS

To investigate the developmental time points at which neurospheres can be generated from NSCs (using the well established neurosphere assay, Reynolds and Weiss, 1996), the ability of cells derived from the embryonic mouse dorsal telencephalon (DT) and spinal cord (SC) to generate neurospheres was assayed. This was done using both bFGF and EGF to allow the greatest chance of obtaining NSCs. At E11.5 and E14.5 a large number of neurospheres can form from the DT, with the greater number of spheres generated at E14.5. At E10.5 a much reduced number of neurospheres can form from the DT. With a 5-fold increase observed between this stage and a day later at E11.5. Most significantly, cells from the mouse DT at E9.5 were unable to generate neurospheres (Figure 3:2:1). In a similar manner, cells derived from the mouse SC gave rise to a large number of neurospheres upon dissociation at E11.5 and E14.5, with the greater number of spheres formed at E11.5. The number of neurospheres forming at E10.5 was less than 20% that formed at E11.5. At E9.5, similar to the DT, the SC was unable to form neurospheres (Figure 3:2:2). Upon differentiation of E14.5 DT and E11.5 SC neurospheres, the three main cell types of the CNS, neurons, astrocytes and oligodendrocytes could form. This confirmed that the neurospheres from each of these regions fulfils the definition of a neurosphere formed from a NSC (Figure3:2:3).

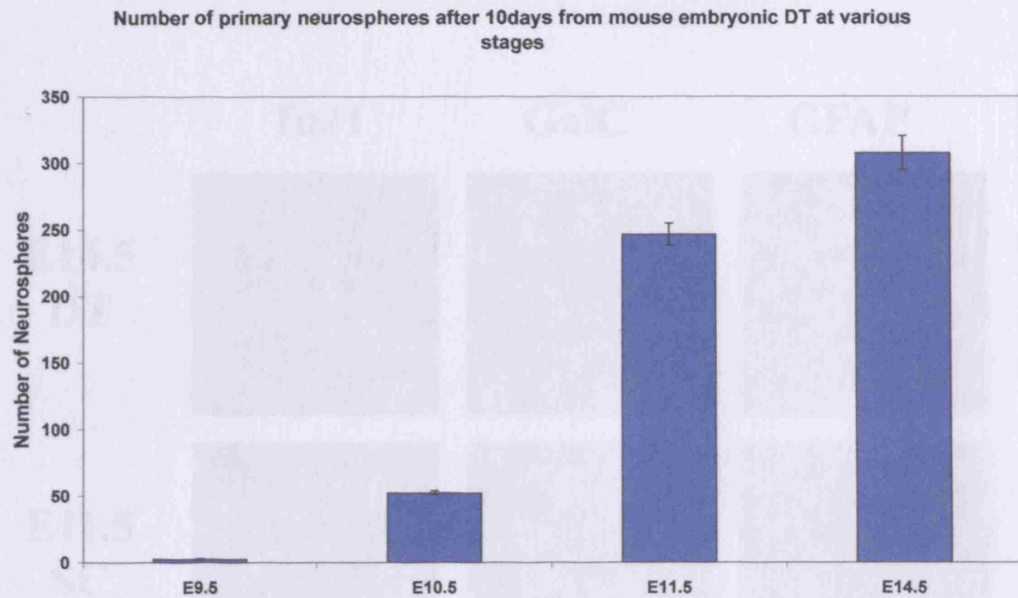


Figure 3:2:1 The dorsal telencephalon (DT) tissue at each stage was dissociated to single cells, then plated at a density of 5×10^4 cells/ml, after 10days the number of neurospheres that formed were counted. At E9.5 the mouse DT was unable to generate neurospheres, by E10.5 a small number of neurospheres were able to form. At E11.5 and E14.5 many neurospheres could form, with the larger number of spheres forming at E14.5. Bars indicate standard error.

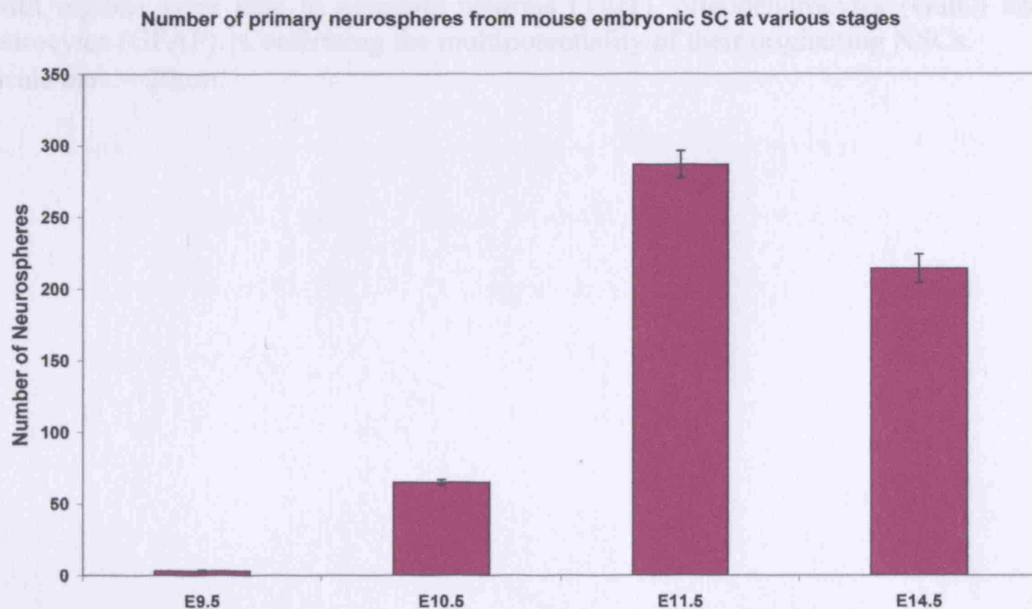


Figure 3:2:2 The spinal cord (SC) tissue at each stage was dissociated to single cells, then plated at a density of 5×10^4 cells/ml, after 10days the number of neurospheres that formed were counted. At E9.5 the mouse SC was unable to generate neurospheres, by E10.5 a small number of neurospheres were able to form. At E11.5 and E14.5 many neurospheres could form, with the larger number of spheres forming at E11.5. Bars indicate standard error.

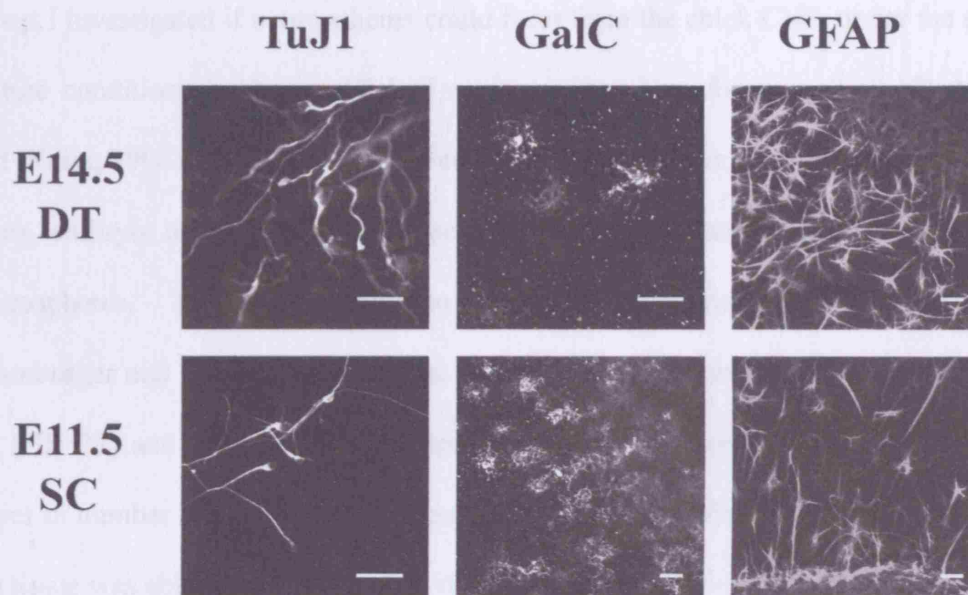


Figure 3:2:3 Neurospheres from mouse E14.5 dorsal telencephalon (DT) and E11.5 spinal cord (SC) (the time points that the greater number of spheres form from each CNS region) were allowed to differentiate in the absence of growth factors for 5 days, they were then fixed and immunohistochemistry carried out. Neurospheres from both regions were able to generate neurons (TuJ1), oligodendrocytes (GalC) and astrocytes (GFAP). Confirming the multipotentiality of their originating NSCs. Scale bars = 20µm.

3:2:2 Establishing neurosphere formation from the embryonic chick CNS.

Next I investigated if neurospheres could form from the chick CNS, under the same culture conditions as the established mouse neurosphere forming assay (Reynolds and Weiss, 1996). To do this, single cells were cultured from the DT and SC of the chick embryo at various developmental time points, and challenged to form neurospheres. The DT was able to give rise to the most neurospheres at E6 (Hamburger and Hamilton (HH) stage 28-29, Hamburger and Hamilton, 1951). At E4 (HH 23) and E5 (HH 26) neurospheres could form, however there were many fewer in number compared to E6 (Figure 3:2:4). At the earlier stage of E3 (HH 18), the tissue was able to give rise to few if any neurospheres. In a similar manner to the DT, the SC at E6 could generate the greater number of neurospheres (Figure 3:2:5). However unlike the DT, the SC at an earlier developmental time point than E6 (E3 to E5) was unable to give rise to any neurospheres. As the formation of neurospheres had not previously been characterised from chick CNS, this study has defined the spheres forming at E6 from both the DT and SC as neurospheres by their ability to: 1) give rise to neurons (TuJ1), oligodendrocytes (GalC) and astrocytes (GFAP) upon differentiation (Figure 3:2:6), conferring multipotentiality; as well as 2) give rise to secondary neurospheres (Figure 3:2:7) upon dissociation of primary neurospheres, conferring self renewal.

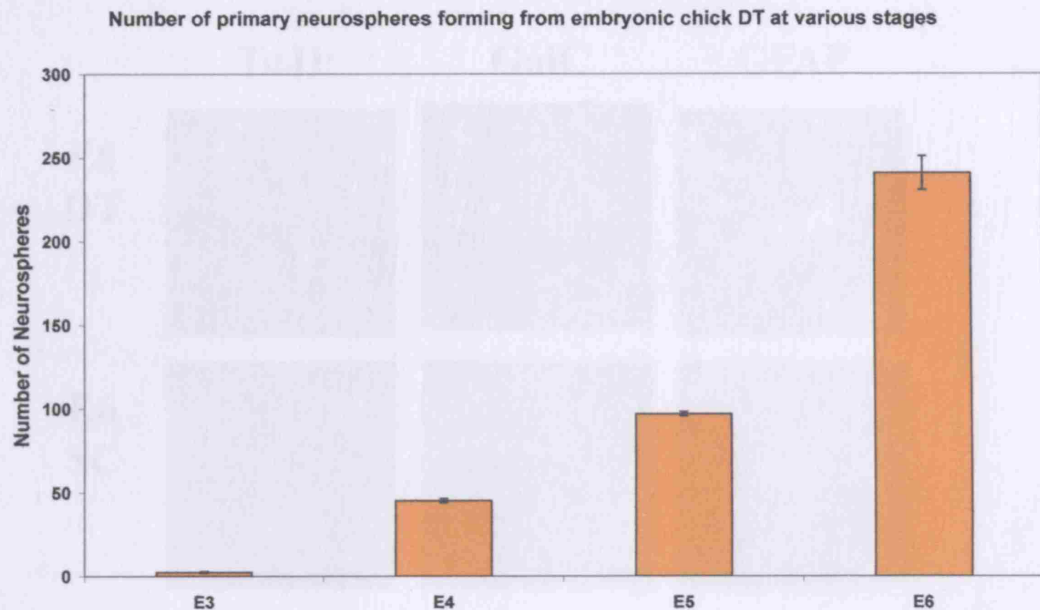


Figure 3:2:4 The ability of cells from the chick dorsal telencephalon (DT) to form neurospheres at various stages was assessed. Cells were plated at a density of 5×10^4 cells/ml, and the number of neurospheres that formed were counted after 10 days. At E3 the chick DT was able to generate few if any neurospheres, at E4 and E5 a small number of neurospheres were able to form. By E6, many neurospheres were generated. Bars indicate standard error.

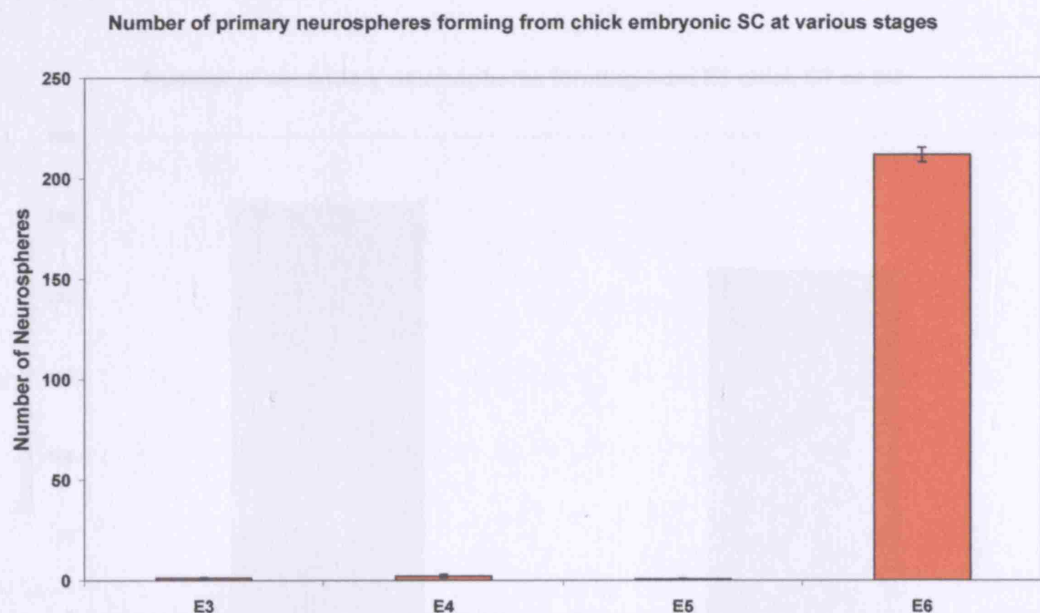


Figure 3:2:5 The ability of cells from the chick spinal cord (SC) to form neurospheres at various stages was assessed. Cells were plated at a density of 5×10^4 cells/ml, and the number of neurospheres that formed were counted after 10 days. At E3, E4 and E5 cells from the chick spinal cord (SC) was able to generate few if any neurospheres, by E6, many neurospheres were generated. Bars indicate standard error.

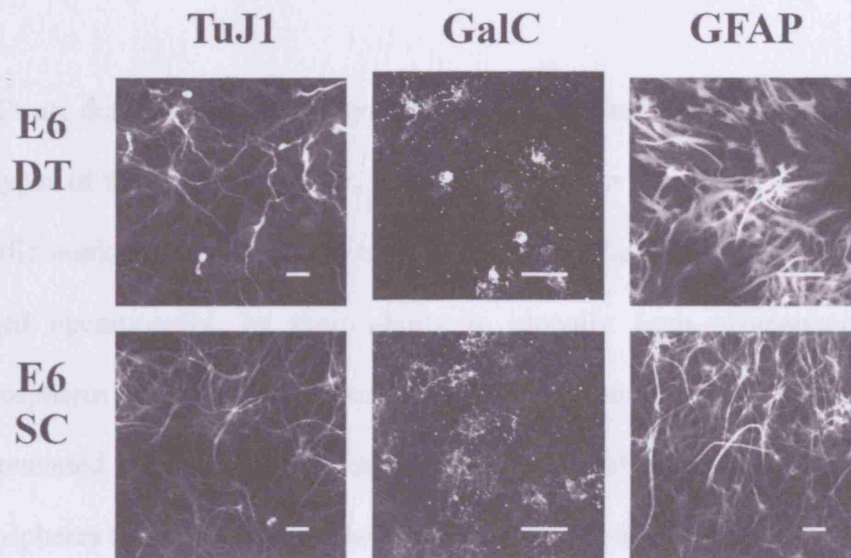


Figure 3:2:6 Neurospheres from chick E6 dorsal telencephalon (DT) and spinal cord (SC) (the time points that the greater number of spheres form from each CNS region) were allowed to differentiate in the absence of growth factors for 5days, they were then fixed and immunohistochemistry carried out. Neurospheres from both regions were able to generate neurons (TuJ1), oligodendrocytes (GalC) and astrocytes (GFAP), upon differentiation. Confirming the multipotentiality of their originating NSCs. Scale bars= 20 μ m.

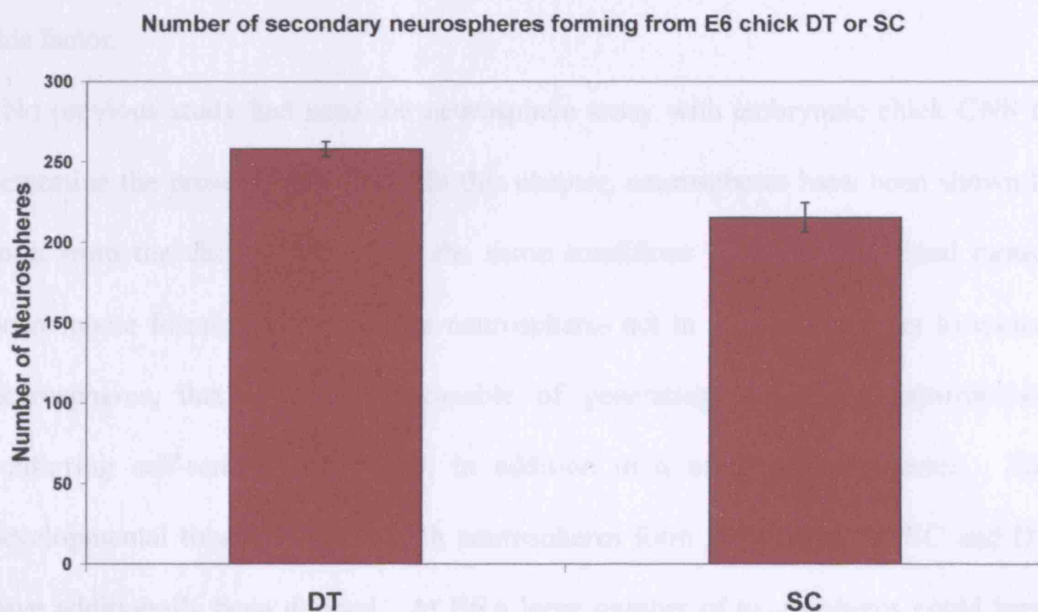


Figure 3:2:7 Upon dissociation of primary neurospheres from E6 chick dorsal telencephalon (DT) and spinal cord (SC) to single cells, and re-plating at a density of 5×10^4 cells/ml, both regions were able to give rise to secondary neurospheres. Counts here indicate the number of secondary neurospheres formed after 10days in culture, and indicate that the chick NSCs are capable of self-renewal. Bars indicate standard error.

3:3 Discussion

NSCs are defined by their ability to proliferate, self renew and give rise to the main cell types of the CNS. However, very little is known about their behaviour *in vivo*. Specific markers for NSCs have not yet been identified, therefore NSCs have to be defined operationally, by their ability to clonally form neurospheres *in vitro*. Neurospheres are floating, spherical structures composed of NSCs, NPCs and differentiated cell types. Previously no study had defined the time points at which neurospheres could form from the mouse embryonic DT and SC. This chapter has shown that neurospheres cannot form at E9.5 from either the DT or SC, and it was not until E11.5 that significant numbers of neurospheres are generated from both regions. These data suggest that a factor necessary for NSC proliferation (neurosphere formation) is absent at E9.5, and is only induced/ activated between E10.5-E11.5. In the following chapters evidence will be provided for the identity of this factor.

No previous study had used the neurosphere assay with embryonic chick CNS to determine the presence of NSCs. In this chapter, neurospheres have been shown to form from the chick CNS, under the same conditions as the well defined mouse neurosphere forming assay. These neurospheres act in a similar manner to mouse neurospheres, that is they are capable of generating secondary neurospheres, conferring self-renewal capability, in addition to a multipotent character. The developmental time points at which neurospheres form from the chick SC and DT have additionally been defined. At E6 a large number of neurospheres could form from both sources, however fewer spheres could form from the DT at E4, and E5, with E3 DT being unable to form any neurospheres. The SC was not able to give rise to a significant number of neurospheres before E6. The use of the chick in

addition to the mouse as a model to study neurosphere formation, allows the use of the well defined technique, *in ovo* electroporation, which will be utilized in the following chapters to further understand the factors essential for neurosphere formation from NSCs.

The average number of neurospheres formed from 5×10^4 cells derived from the mouse and chick CNS tissue at various developmental stages, is summarised in the following tables.

Mouse

| Stage | Dorsal Telencephalon | Spinal Cord |
|-------|----------------------|-------------|
| E9.5 | 2.5 | 3.17 |
| E10.5 | 52.3 | 65 |
| E11.5 | 246.3 | 286.7 |
| E14.5 | 307.5 | 214.17 |

Chick

| Days | HH Stage | Dorsal Telencephalon | Spinal Cord |
|------|----------|----------------------|-------------|
| E3 | 18 | 2.5 | 1.25 |
| E4 | 23 | 45.25 | 2.25 |
| E5 | 26 | 97 | 0.75 |
| E6 | 28 - 29 | 240.75 | 211.75 |

Chapter 4

The Role of *Sox9* in Neural Stem Cells

Chapter 4

The role of *Sox9* in neural stem cells

4:1 The *Sox* genes

The SOX (SRY-related HMG box) proteins, contain a single HMG box type of DNA-binding domain, and are highly conserved across evolution (Pevny and Lovell-Badge, 1997). The SOX proteins are defined by having an HMG box with at least 50% homology with that of mouse SRY (the mammalian sex determining gene). The *Sox* gene family within mammals consists of 20 members, and can subsequently be divided into ten subfamilies according to their degree of sequence identity both within and outside the HMG box (Wilson and Koopman, 2002). SOX proteins play a crucial role in embryonic development by activation or repression of their target genes. They can form multi-protein complexes with gene promoters or enhancers, and their specific partners within each of the tissues in which they are expressed will determine the outcome (Wilson and Koopman, 2002). The HMG box of the SOX proteins recognises specific nucleotide sequences (with a consensus site being AACAAAT), binding the minor groove and bending the DNA to an acute angle (Nagai, 2001; Ferrari et al., 1992).

4:2 Role of *SoxB1* genes in neural stem cells

The *SoxB1* family comprises *Sox1*, *Sox2* and *Sox3*, which share more than 90% homology within their HMG box DNA binding domains. *Sox2* RNA is first detected in the mouse at the morula stages (E2.5), as well as in the blastocyst (E3.5) in the inner cell mass (ICM). By E7-7.5, *Sox2* expression becomes largely restricted to the early anterior neuroectoderm. By E9.5 *Sox2* is expressed throughout the brain, neural tube,

sensory placodes, branchial arches, and endoderm of the gut (Avilion, et al., 2003; Wood and Episkopou, 1999). *Sox2* is believed to be essential for early CNS development, based on its expression, as well as functional data. *Sox2* homozygous null mice are unable to form the primitive ectoderm, and die just after implantation (Avilion et al., 2003). *Sox2* ^{β geo} mutants were generated by gene targeting, where the *Sox2* open reading frame (ORF) was replaced with a β geo cassette encoding a β -galactosidase and neomycin resistance fusion protein (Avilion et al., 2003). Therefore allowing the visualisation of the activity of the *Sox2* locus through β -galactosidase activity, and the selection of *Sox2* ^{β geo} positive cells, through neomycin resistance. *Sox2* ^{β geo} heterozygous mice are viable. Compound heterozygotes were generated with the *Sox2* ^{β geo} mice and a regulatory mutant allele (with a neural cell-specific enhancer deleted) (Ferri et al., 2004). These compound heterozygotes exhibit cerebral malformations and proliferative defects of adult NSCs/NPCs. Ferri et al. (2004) conclude that *Sox2* plays a vital role in the maintenance of neurons.

In addition, *Sox2* has been found to label NSCs, although not exclusively, both in the adult and the embryo (Komitova and Erikson, 2004). A transgenic mouse line was generated by homologous recombination, where the *Sox2* open reading frame was replaced with EGFP (Ellis et al., 2004). Therefore, EGFP expression recapitulates endogenous SOX2 expression. SOX2 expression was visualised in proliferating neural progenitor cells (NPCs) in the CNS during embryogenesis. In the adult CNS, SOX2 was expressed in known NSC niches, including the subventricular zone (SVZ), the dentate gyrus, ependymal layer of the lateral ventricles, and the ependyma of the central canal. SOX2-EGFP was also seen to be expressed in neurospheres generated from both embryonic and adult CNS tissue (Ellis et al., 2004). However, *Sox2* has been found to be expressed in postmitotic neurons, e.g. pyramidal cells of the cortex, striatal neurons

and neurons of the thalamus (Wegner and Stolt, 2005), therefore is not restricted to undifferentiated NSCs/NPCs.

Sox1 and *Sox3* null mutants are viable. *Sox1* is expressed after the formation of the neural plate, and *Sox3* is expressed in the epiblast and extraembryonic ectoderm (Wood and Episkopou, 1999). *Sox1* homozygous null mice show no significant defects in CNS development, although these animals have defects in lens development and suffer from epilepsy due to a neuronal defect in the ventral forebrain and olfactory cortex hyperexcitability (Malas et al., 2003). *Sox3* homozygous null mice exhibit craniofacial abnormalities as well as reduced size and fertility (Rizzoti et al., 2004).

Sox1, as well as *Sox2* is thought to label both embryonic and adult NSC. To study this, *Sox1*-GFP transgenic mice were generated by gene targeting, in which an enhanced green fluorescent protein (EGFP) reporter was inserted into the *Sox1* gene. In these mice, GFP recapitulates *Sox1* expression. GFP was visualised in the embryo throughout the CNS (from E8.5 onwards) as well as in the lens. In adult mice GFP was visualised predominantly in the dentate gyrus subgranular layer (Aubert et. al., 2003).

In addition to *Sox1* and *Sox2* being expressed in known NSC niches both in the adult and the embryo, it has also been shown that *Sox1* and *Sox2* can initiate neural differentiation. For example, ectopic expression of *Sox2* (along with FGF) in *Xenopus*, initiates neural differentiation of ectoderm. Likewise, injection of a dominant negative *Sox2* construct in *Xenopus* inhibits neural differentiation (Pevny and Placzek, 2005). In addition, when *Sox1* was overexpressed in P19 cells (mouse embryonic carcinoma cells), a neuroectodermal fate was promoted rather than mesodermal or ectodermal fates (Pevny et al., 1998). The inhibition of SOX2 signalling leads to the delamination of neural progenitors from the ventricular zone and exit from the cell cycle, a phenotype that can be rescued by co-expression of SOX1 (Graham et al., 2003). In the chicken, *Sox2* and *Sox3* are expressed in the neural epithelium of the developing CNS (HH 10-

34). *Sox11* (group C) is also expressed in the neural epithelium, however it is transiently up-regulated in maturing neurons leaving the neural epithelium. According to the pattern of expression of these *Sox* genes provides further evidence that *Sox* genes play a role in neural development and indicates that the maturation of neuronal populations could involve ‘switching’ of *Sox* gene expression (Uwanogho et al., 1995). Additionally, a study using chick *in ovo* electroporation, indicate that *Sox1*, *Sox2* and *Sox3* can maintain neural cells in an undifferentiated state, and suppression of *Sox1-3* expression allows for the generation of neurons (Bylund et al., 2003). Together, these data indicate that *SoxB1* genes are essential for early neural fate decisions.

4:3 The role of *Sox9* in stem cells

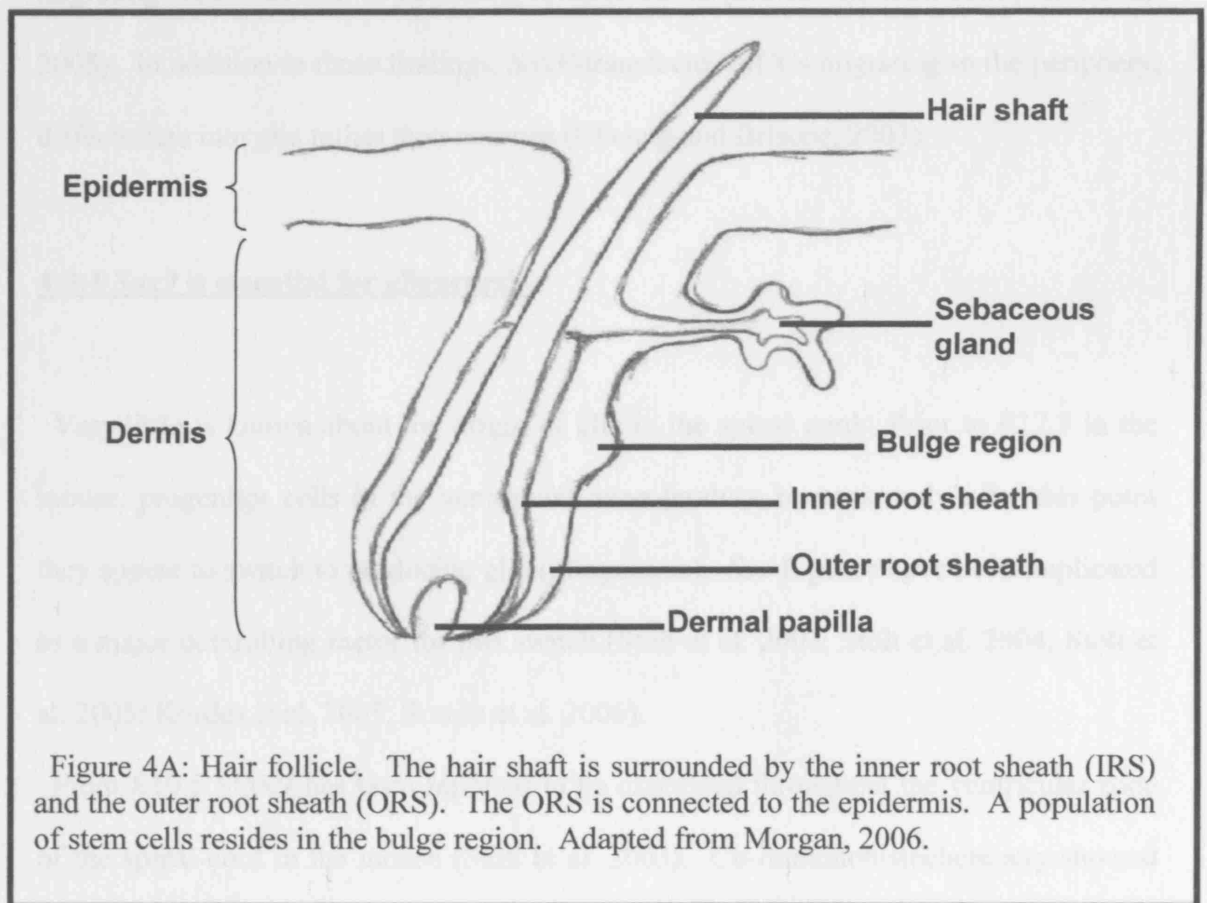
Sox9 is a member of the *Sox* gene family, subgroup E, along with *Sox8* and *Sox10*. The first evidence for the role of *Sox9* in development comes from the study of human Campomelic dysplasia (CD) patients. CD results from heterozygous mutations of the *SOX9* gene (Foster et al., 1994). Often death occurs neonatally due to respiratory problems, but life expectancy varies widely depending on the severity of the phenotype (Akiyama et al., 2004). CD patients exhibit skeletal malformation amongst other developmental abnormalities (Giordana et al., 2001; Houston et al., 1983; Maroteaux et al., 1971). Two-thirds of XY CD individuals show sex reversal (Houston et al., 1983; Wagner et al., 1994). It was discovered that *SOX9* plays a vital role in the expression of *Col2a1* (a chondrocyte-specific marker) as well as the expression of an anti-Mullerian hormone, secreted from the Sertoli cells for male sex differentiation (Bell et al., 1997; De Santa Barbara et al., 1998). *Sox9* is therefore best known for its roles in male sex determination (Chaboissier et al., 2004) and in chondrogenesis (Akiyama et al., 2002; Bell et al., 1997). This role was further indicated by a study using a mouse transgenic

line, *Sox9* null embryonic stem cells were unable to undergo chondrogenesis *in vitro*, in addition, the ectopic expression of *Sox9* induced the endogenous activation of *Col2a1* in transgenic mice (Akiyama et al., 2002).

CD patients also exhibit developmental defects in the inner ear, brain, pancreas and heart (*SOX9* is expressed in all of these areas) (Houston et al., 1983; Mansour et al., 2002). As well as skeletal malformation and sex reversal, symptoms can also include, deafness, cleft palate and scoliosis, indicating that there could also be defective development of cranial neural crest cells and of the notochord (Spokony et al., 2002; Cheung and Briscoe, 2003). Frequently, patients suffer from macrocephaly with dilations of the lateral ventricles, and in around 25% of patients the olfactory tracts and bulbs are missing (Mansour et al., 1995). The CNS defect observed in CD patients, indicates a possible role for *SOX9* in the development of the CNS.

Sox9 is also required for normal development and maintenance of hair (Vidal et al. 2005). Hair undergoes continuous cycling throughout adult life. During mouse development, hair begins to form at E14.5 by inducing the ectodermal epithelium to thicken forming 'placodes'. These epithelial cells in close proximity to the dermal papilla constitute the highly proliferative matrix of the hair bulb. Matrix cells divide and move upwards whilst differentiating into three cell layers of the hair shaft (medulla, cortex and cuticle) and the epithelial cells around the hair shaft. The inner root sheath (IRS) is made up of three layers of cells. The outer root sheath (ORS) surrounds the IRS, and is connected directly to the basal layer of the epidermis. A population of stem cells resides in a region known as the bulge (Blanpain et al., 2004). The bulge is contiguous to the ORS and located near the erector pili muscle (Figure 4A). The growth of new hair requires the stimulation of the bulge stem cells, which then leave the bulge, migrate toward the matrix, proliferate and differentiate forming the new shaft of hair. The signal inducing new hair growth is believed to involve Sonic hedgehog (SHH)

and Wnt in some part. Vidal et al. (2005) showed that SOX9 was expressed and active in the hair placodes at the time of their induction (E14.5) as well as being expressed and active in the bulge region and ORS. Additionally, they reported that when *Sox9* was specifically inactivated in the skin of transgenic mice, hair failed to form normally (alopecia). Vidal et al. (2005) also provided evidence that SHH signalling is required for SOX9 expression in the skin.



SoxE genes are expressed in the neural crest, with *Sox9* being expressed prior to that of premigratory neural crest markers (Cheung and Briscoe, 2003). Neural crest cells (NCCs) are a transient migratory stem cell population, originating from the dorsal neural folds bordering the neural plate and epidermal ectoderm. Mammalian NCCs can be isolated and cultured clonally. They are multipotent and capable of self-renewal, providing evidence that these NCCs are bonafide stem cells (Stemple and Anderson,

1992). Upon induction, NCCs delaminate from the neural tube and migrate into the periphery, differentiating into a variety of cell types e.g., peripheral nervous system (PNS) neurons and glia, melanocytes and craniofacial skeletal and connective tissue (Cheung and Briscoe, 2003; McKeown et al. 2005). Upon the forced expression of *Sox9* within the neural tube, ectopic neural crest differentiation is induced (Cheung and Briscoe, 2003). Similarly, over expression of *Sox10* in the neural tube induced migrating NCCs, as well as inhibiting differentiation (McKeown et al. 2005; Kim et al. 2003). In addition to these findings, *SoxE*-transfected NCCs migrating in the periphery, differentiate into glia rather than neurons (Cheung and Briscoe, 2003).

4:3:1 Sox9 is essential for gliogenesis

Very little is known about the origin of glia in the spinal cord. Prior to E12.5 in the mouse, progenitor cells in the ventricular zone produce neurons, only after this point they appear to switch to producing glia (gliogenesis). *Sox E* genes have been implicated as a major controlling factor for this switch (Stolt et al. 2003; Stolt et al. 2004; Stolt et al. 2005; Kordes et al. 2005; Sottile et al. 2006).

From E10.5 SOX9 has been reported to be expressed throughout the ventricular zone of the spinal cord in the mouse (Stolt et al. 2003). Co-immunohistochemistry showed that SOX9-expressing cells in the ventricular zone were nestin-positive and brain fatty acid binding protein (B-FABP) (a marker of radial glia) positive. SOX9 was additionally expressed in radial glia. Later on in embryogenesis as well as in the adult, all GFAP (glial fibrillary acidic protein) positive astrocytes stained positive for SOX9 in the spinal cord (Stolt et al. 2003). A small percentage of SOX9 positive cells in the embryonic spinal cord were also positive for oligodendrocyte progenitor markers (PDGF receptor α). However, Stolt et al. (2002) demonstrated that neurons and

endothelial cells of the embryonic spinal cord were negative for SOX9. Stolt et al. (2005) also specifically ablated *Sox9* in the CNS, using a floxed *Sox9* allele (Akiyama H. et al. 2002), combined by breeding with a nestin-cre transgene, which is expressed throughout the early CNS. Stolt et al. (2005) found that spinal cord development appeared normal until E14.5, but by E16.5 the size appeared greatly reduced. By E18.5 apoptosis had increased leading to degeneration of the spinal cord. However, no alteration in neuron distribution was observed. In contrast an overall reduction of 95% of oligodendrocytes in *Sox9* null spinal cords, and additionally a 30% increase in motor neuron number was revealed relative to wild type embryos. Virtually no astrocytes (S100 β -positive) were observed in the null mutant spinal cords. Glast (glial glutamate transporter) positive radial glia/astrocytes were also severely reduced. However, at later stages of embryogenesis, oligodendrocyte progenitor numbers recovered slightly, explained as a result of compensatory actions of the closely related *Sox10* and *Sox8*. Astrocyte numbers did not recover at later stages. These mice with *Sox9* ablated throughout their CNS, die perinatally.

Using a *Sox10* β -galactosidase mouse line (*Sox10* expression is recapitulated by LacZ staining), Stolt et al (2002) showed that SOX10 was expressed in oligodendrocyte precursors at E12.5 in the ventricular zone in the (motor neuron progenitor) pMN domain of the ventral spinal cord. SOX10 expression continued in migrating and proliferating oligodendrocyte progenitors and was subsequently down-regulated in differentiating myelin-forming oligodendrocytes. SOX10 was not visible in terminally differentiated oligodendrocytes. Using mouse lines *Sox10*^{LacZ} and *Sox8*^{LacZ} and by co-immunohistochemistry, it was shown that both *Sox8* and *Sox10* cooperate to influence the terminal differentiation of oligodendrocytes (Stolt et al. 2004). Moreover, the same group later showed that SOX8 is expressed in the developing spinal cord just after SOX9 but prior SOX10. *Sox8* probably induces the remaining oligodendrocyte

progenitors in the *Sox9* null mice, as oligodendrogenesis was virtually absent in spinal cords deficient for both *Sox8* and *Sox9* (Stolt et al. 2004). *Sox8* null mice suffer no obvious defects, other than weight loss (Sock et al., 2001), whereas *Sox10* null mice die either before or at birth (Britsch et al., 2001).

In agreement with findings from Stolt et al. (2005) in the spinal cord, Sox E genes have also been found to be expressed by glial cells in the developing chick and mouse brain (Kordes et al. 2005; Sottile et al. 2006). SOX9 shows particularly high expression in known proliferative, stem cell-containing zones of the brain, e.g., the ventricular zone. All *SoxE* genes were expressed in the Purkinje layer as well as in the ventricular zone of the lateral ventricles of the forebrain. Co-staining of SOX9 with calbindin (a Purkinje cell marker) and vimentin (a marker of Bergmann glia) provided evidence that SOX9 was expressed in Bergmann glia (a type of radial glia). In addition, the location of SOXE immunopositive cells in proliferative zones known to contain uncommitted precursors and stem cells, suggested that these genes may play a role in the multipotent stem cell state (Kordes et al. 2005).

Further evidence of a role for *Sox9* in the neural stem cell state emerges from a study looking at its expression pattern in the adult mouse brain (Sottile et al. 2006). SOX9 was found to have a similar expression pattern to SOX1 and SOX2 in specific regions, both of which are genes already implicated as neural stem cell markers. For example, SOX9 was expressed in the subdentate gyrus region of the hippocampus and subventricular zone, both areas where adult NSC niches have been described, and strong expression in the lining of the ventricles. In addition to the expression of SOX9 in the ventricular zone, it is expressed in the Bergmann glia of the cerebellum (Kordes et al. 2005; Sottile et al. 2006). These Bergmann glia also express SOX1 and SOX2, although the former is not necessary for their development, possibly due to functional redundancy (Sottile et al. 2006).

4:3:2 Evidence that glia can also function as neural stem cells

In the CNS, glial cells outnumber neurons. The two main types of glial cells in the CNS are astrocytes and oligodendrocytes (Kandel et al., 2000). Radial glia appear at the onset of neurogenesis in the ventricular zone. They are characterised by an ovoid cell body (near the ventricular surface) and are bipolar in form. Some of their processes terminate in end-feet, which project to the ventricular surface and the pia mater (Doetsch 2003; Noctor et al. 2002). Radial glia share many glial characteristics, for example, they have many intermediate filaments, glycogen granules condensed at the end-feet and express many glial markers such as GFAP, RC2, vimentin, nestin, BLBP (Brain lipid binding protein), GLAST and tenascin (Doetsch 2003; Anthony et al. 2004; Parnavelas and Nadarajah 2001). Although, NSCs were thought to be undifferentiated cells, much evidence has arisen that radial glia, with many differentiated glial characteristics, are indeed neural stem cells, that is they can give rise to neurons and glia (Anthony et al. 2004; Ever and Gaiano 2005; Parnavelas and Nadarajah, 2001; Ganat et al. 2006; Malatesta et al. 2003).

Neurons in many regions of the brain derive from radial glia as shown by Cre/loxP fate mapping and clonal analysis (Anthony et al. 2004). BLBP was used in these studies as an exclusive marker of radial glia and astrocytes, and showed that virtually all neuronal populations in the mouse brain derive from BLBP-expressing progenitors, with most of the telencephalic neurons being derived from BLBP positive radial glia (Anthony et al. 2004).

McMahon and McDermott (2007) isolated radial glia from E14.5 rat spinal cords (by incubating with Mab2F7, a neuronal cell surface antigen), and marked these cells with a tracking dye to enable detection after transplantation. These cells were injected into host embryos at various embryonic stages, as well as post natal day (P) 1 and 7 (P1 and

P7) into the telencephalic vesicle or 4th ventricle of the embryos. They found that radial glia migrated further in younger hosts, but differentiated earlier in older hosts. Most of the glia differentiated into astrocytes, however, some gave rise to neurons in the spinal cord. The latter were not found in the brain, which perhaps reveals regional specificity since the transplanted radial glia, originated from the spinal cord.

There are various subtypes of radial glia both within and across different brain regions (Kriegstein and Gotz 2003; Malatesta et. al. 2003). These subtypes differ in their potential to form different cell types, their expression of growth factors and transcription factors (Kriegstein and Gotz 2003) leading to the possibility that radial glia could play a major role in the regionalisation of the developing CNS. Malatesta et. al. (2003) used Cre/loxP *in vivo* fate mapping studies to reveal radial glial fate in different regions of the brain. Using human GFAP Cre-mediated recombination, they showed that virtually all cortical projection neurons were generated by radial glia, but ventral telencephalic interneurons were not. Few neurons in the basal ganglia originate from radial glia. *In vitro* they showed a difference in the potential of radial glia of the dorsal telencephalon compared to those of the ventral telencephalon (Malatesta et al., 2003).

Further evidence that radial glia fulfil the characteristics of a NSC, is provided by the presence of radial glia in the adult NSC niche (Merkle et al. 2004; Ganat et al. 2006). Merkle et al. (2004) permanently labelled striatal radial glia, by injecting an adenovirus to tag a restricted population of cells in the lateral wall of the lateral ventricle, in newborn mice and followed their progress. They showed that the radial glia were able to give rise to neurons, oligodendrocytes and astrocytes, including astrocytes of the subventricular zone (SVZ). Labelled cells were also found in the rostral migratory stream (RMS), even in much older mice. They also showed that radial glia of new born mice and of the adult lateral ventricular wall (derived from radial glia) were able to clonally form neurospheres (Merkel et al., 2004).

In a similar manner, Ganat et al. (2006) permanently tagged astroglial cells in the postnatal brain of mice using a transgene containing a tamoxifen inducible Cre recombinase driven by regulatory sequences from human *GFAP*. Initially these tagged cells were quiescent astroglia in the SVZ and dentate gyrus (DG). They also expressed LeX, a stem cell marker. After four weeks, the tagged cells gave rise to proliferating progenitors, expressing neuronal markers in the SVZ and DG, and subsequently the tagged cells of these GFAP lineages generated mature neurons of the olfactory bulb and DG, as well as some in the cerebral cortex. In the cerebral cortex, mature oligodendrocytes and astrocytes were also derived from GFAP expressing astroglial ancestors, proving that at least some glial cells can act as multipotent NSCs.

Given the connection between *Sox9* and some stem cell types, and *Sox9*'s role in gliogenesis, I decided to explore the role of *Sox9* in both the initiation and maintenance of NSCs.

4:4 Results

4:4:1 The expression of SOX E proteins compared to SOX2

Sox2 is known to be expressed as the neural plate forms and throughout the CNS. Previous studies have shown that *Sox2* is required for NSC behaviour (Remboutsika et al., unpublished data). However, as discussed in the previous chapter, neurospheres cannot form from the embryonic dorsal telencephalon (DT) or spinal cord (SC) until E10.5. This coincides with the first appearance of glial cells which have been shown to require *SoxE* genes for their appearance (Stolt et al., 2004).

The expression of SOX9 and SOX10 was therefore compared to SOX2 in the DT and SC of embryos during development (E9.5-E14.5), as well as in the well known NSC niche, the adult (8 week old) subventricular zone (SVZ). SOX9 and SOX10 were first expressed in the dorsal telencephalon and spinal cord at E10.5 and was retained in regions of DT and SC tissue with the ability to generate neurospheres, during later embryogenesis in the P0 spinal cord, as well as in the SVZ of the adult mouse (Figures 4.1 and 4.2). *In vitro*, proliferating neurospheres derived from E14.5 dorsal telencephalon expressed SOX9 and SOX10 in addition to SOX2 (Figure 4.3), expression patterns were similar in proliferating neurospheres derived from E11.5 spinal cord.

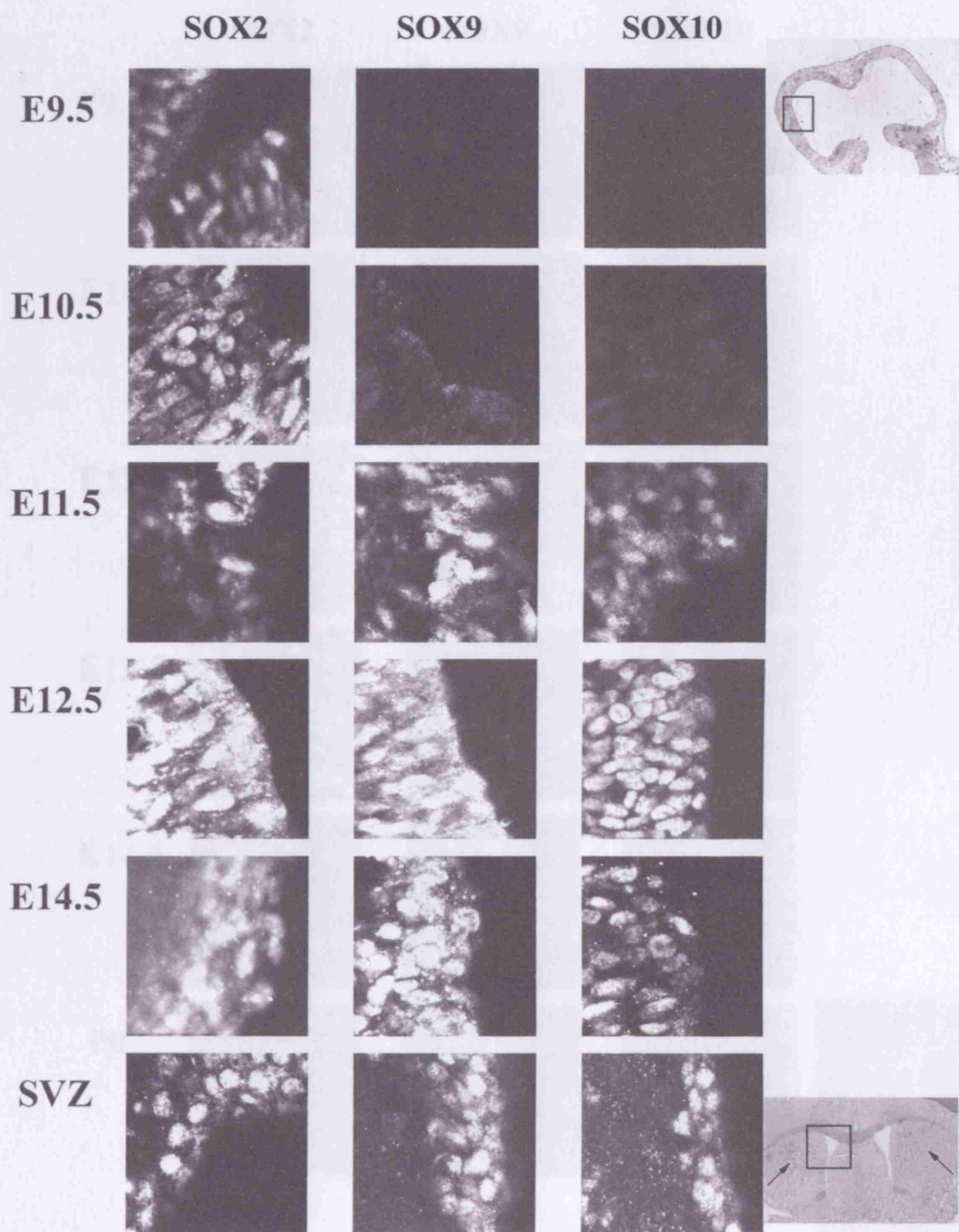


Figure 4.1 Immunohistochemistry on CNS tissue sections was carried out to assess the expression of SOX2, SOX9 and SOX10 in the embryonic dorsal telencephalon from E9.5-E14.5, and the adult (8 week) subventricular zone (SVZ). SOX2 was expressed at all stages, whereas SOX9 and SOX10 were expressed in a small number of cells at E10.5, and strongly expressed from E11.5 onwards. SOX2, SOX9 and SOX10 were expressed in the adult NSC niche, the SVZ.

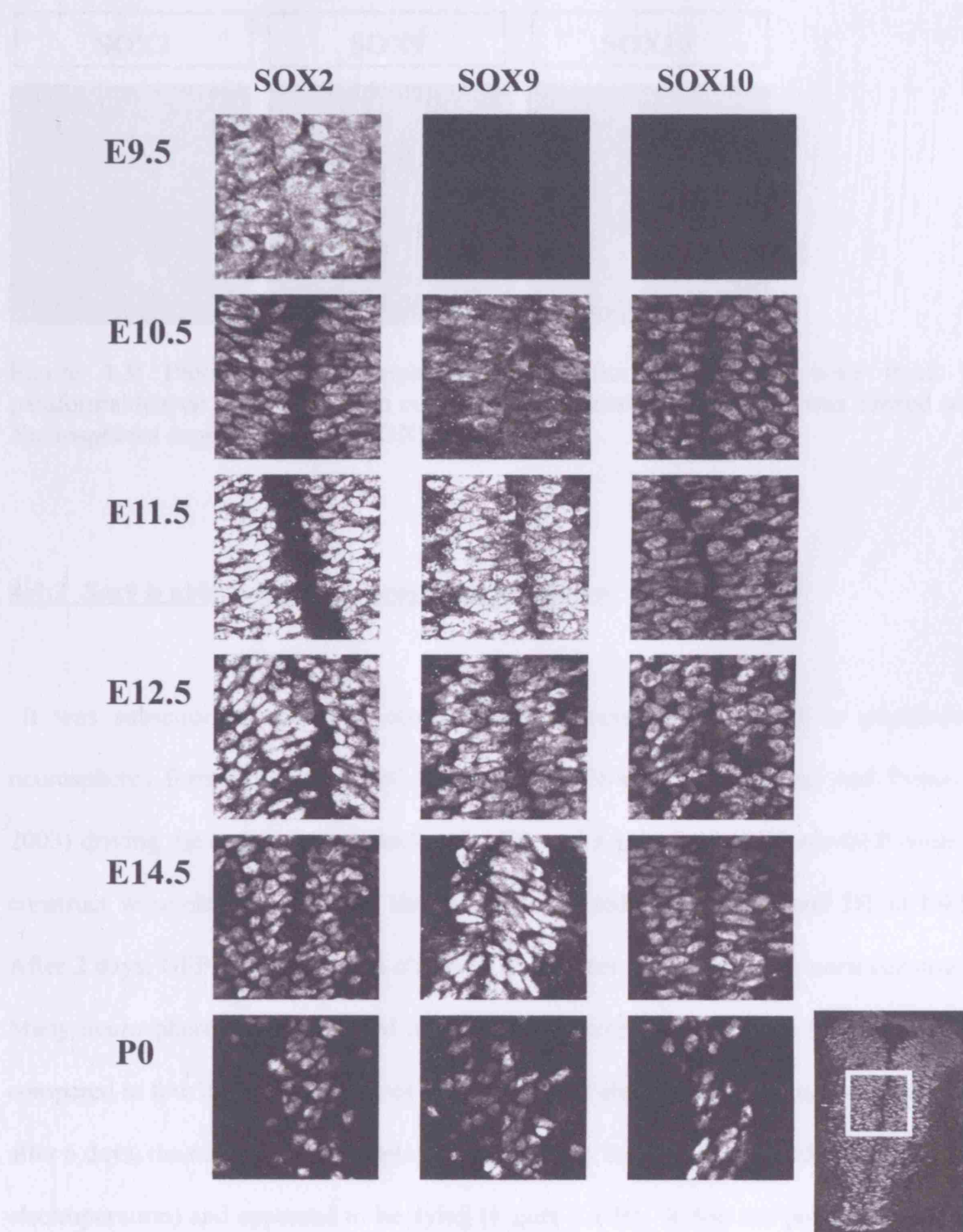


Figure 4.2 Immunohistochemistry on CNS tissue sections was carried out to assess the expression of SOX2, SOX9 and SOX10 in the embryonic spinal cord (SC) from E9.5-E14.5, and the neo-natal (P0) mouse SC surrounding the central canal. SOX2 expression was visible at all stages. SOX9 and SOX10 were expressed from E10.5 onwards, with expression becoming more restricted to the midline as development proceeds.

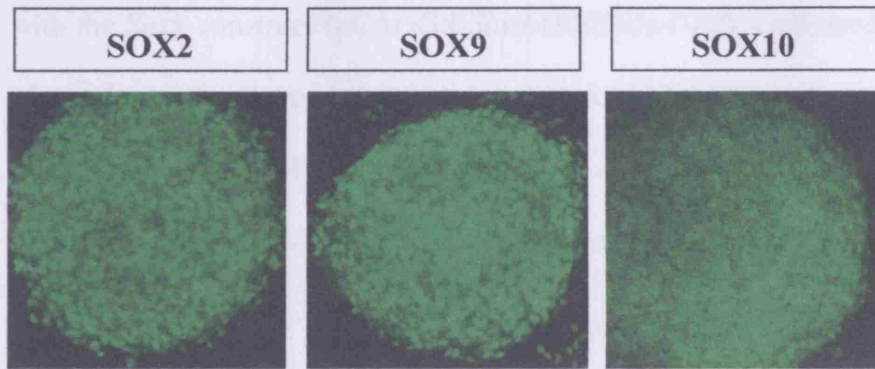


Figure 4.3; Proliferating neurospheres formed from E14.5 DT were fixed in paraformaldehyde after 10 days in culture, and immunohistochemistry was carried out. Neurospheres expressed SOX2, SOX9 and SOX10.

4:4:2 *Sox9* is able to induce neurosphere formation

It was subsequently asked if ectopic *Sox9* expression could lead to precocious neurospheres formation. First, pCAGGS-*Sox9*-IRES-nls-GFP (Cheung and Briscoe, 2003) driving the expression of *Sox9* and GFP, and a pCAGGS-IRES-nls-GFP control construct were electroporated into single cells dissected from the SC and DT at E9.5. After 2 days, GFP expression was observed in cultures transfected with each construct. Many neurospheres were observed in the *Sox9* electroporated cultures (Figure 4.4 A), compared to few if any neurospheres in control, GFP electroporated cultures. However, after 6 days, the number of neurospheres were greatly reduced (compared to 2 days after electroporation) and appeared to be dying (Figure 4.4 B). It was not possible to detect GFP in any cells transfected either with the pCAGGS-*Sox9*-IRES-nls-GFP or pCAGGS-IRES-nls-GFP constructs 4-5 days after the transfection. It therefore seemed likely that *Sox9* is also no longer expressed from the construct several days after the transient transfection.

A similar experiment was performed by transfection of cells isolated at E10.5. An initial increase was observed in neurosphere formation in the cultures electroporated

with the *Sox9* construct (pCAGGS-*Sox9*-IRES-nls-GFP), compared to a fewer number of smaller neurospheres being generated in control cultures (those electroporated with pCAGGS-IRES-nls-GFP) (Figure 4.4 C). However, in this case the increase in neurosphere numbers in *Sox9* electroporated cultures was maintained at least to 6 days (Figure 4.4 D).

Number of neurospheres formed from the dorsal telencephalon at E9.5 and E10.5 following electroporation with pCAGGS-*Sox9*-IRES-nls-GFP compared to controls, two and six days after the transfection

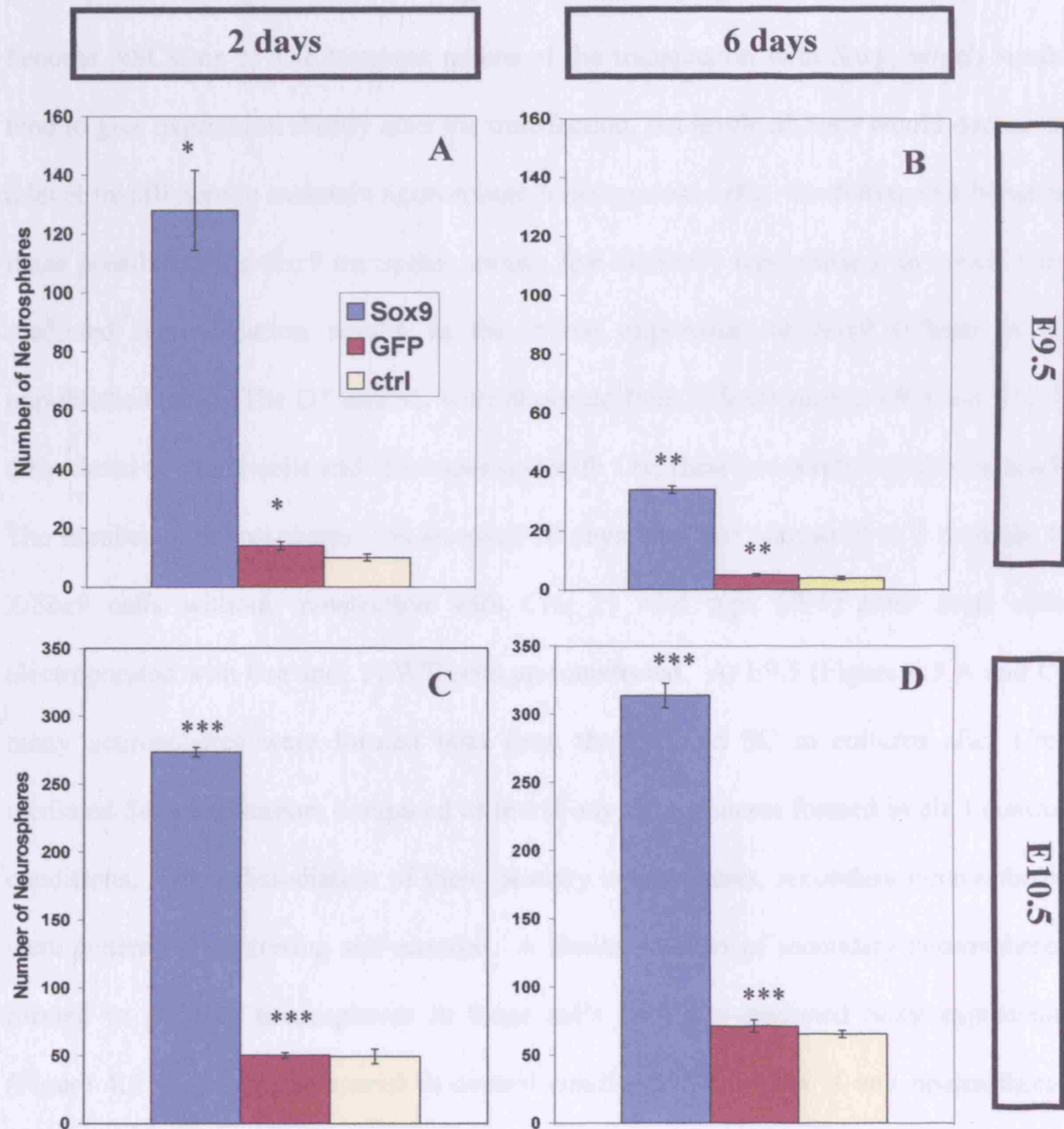
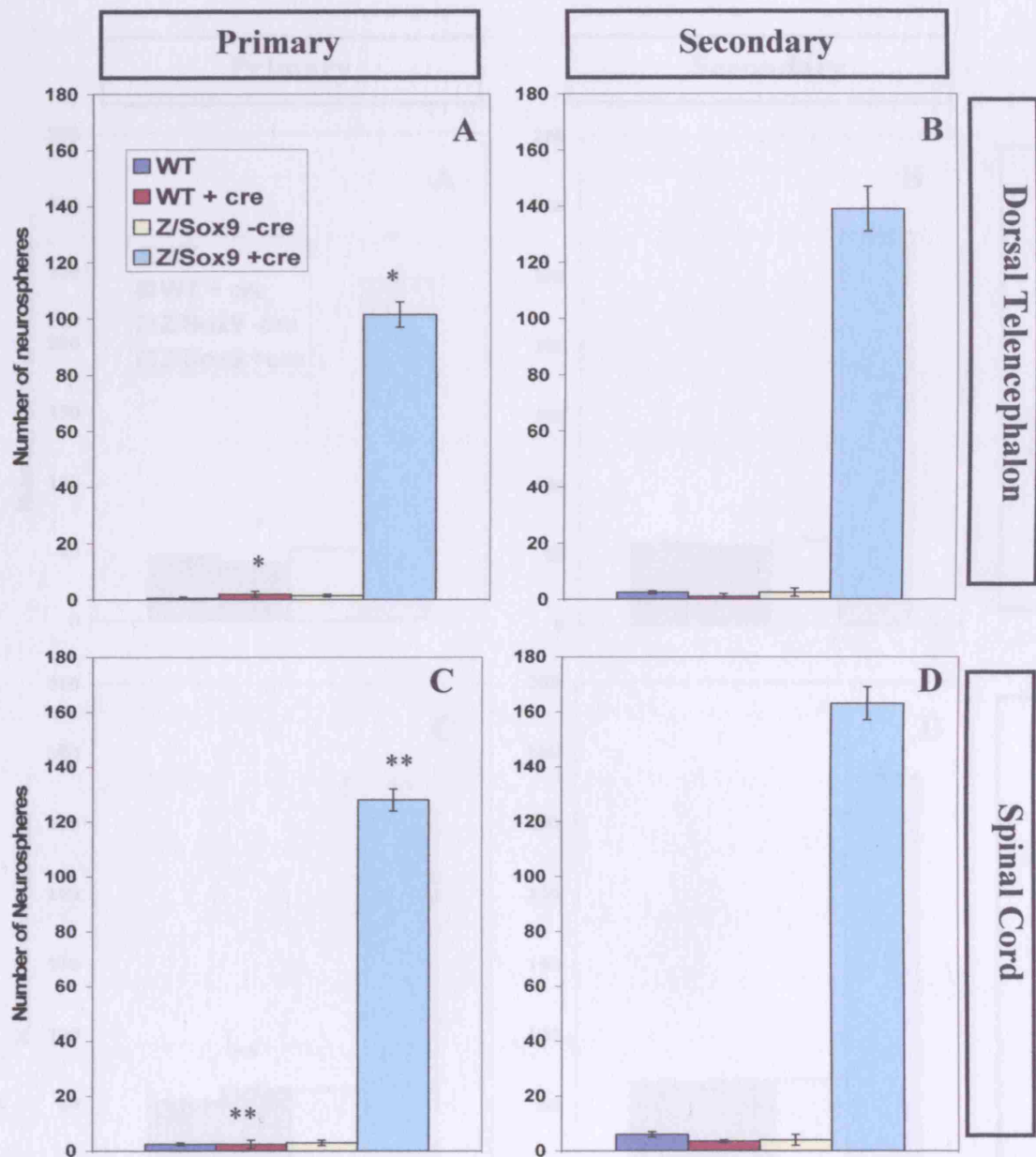


Figure 4.4: E9.5 and E10.5 dorsal telencephalon (DT) tissue was dissociated to single cells and electroporated with either pCAGGS-*Sox9*-IRES-nls-GFP (*Sox9*) or pCAGGS-IRES-nls-GFP (GFP) or no DNA transfection (ctrl). The number of neurospheres were counted 2 then 6 days following the transfection. Very few neurospheres were generated from the E9.5 DT in either of the controls (GFP or ctrl) (A and B), but those transfected with *Sox9* an induction of neurosphere formation was observed (A), this difference was shown to be statistically significant by a Student's t-test * $P < 0.008$. However after 6 days the number of neurospheres formed in *Sox9* transfected cultures had dramatically reduced (B), although this difference was still significant ** $P < 0.003$. At E10.5 transfection with pCAGGS-*Sox9*-IRES-nls-GFP induced a significant increase in neurosphere generation after 2 days (C) and this increase was maintained at least for 6 days (D), compared to the 2 controls (GFP or ctrl). *** $P < 0.0001$. Bars indicate standard error.

The lack of maintenance of neurospheres from E9.5 DT and SC could be due to one of two reasons; 1) An inability of *Sox9* (pCAGGS-*Sox9*-IRES-nls-GFP) to induce neurosphere forming NSCs at this stage, because these progenitors are not competent to become NSCs; or 2) The transient nature of the transfection with *Sox9*, which would tend to give expression shortly after the transfection, but levels of *Sox9* would decline to a level insufficient to maintain neurosphere forming stem cells. To distinguish between these possibilities, a *Sox9* transgenic mouse line (*Z/Sox9*) was utilised, in which Cre-mediated recombination results in the stable expression of *Sox9* (Cheah et al. unpublished data). The DT and SC were dissected from *Z/Sox9* mice at E9.5 and E10.5, dissociated to single cells and electroporated with Cre, therefore stably expressing *Sox9*. The number of neurospheres was assessed 10 days later and compared to 3 controls 1) *Z/Sox9* cells without transfection with Cre; 2) wild type (WT) litter mate cells electroporated with Cre and; 3) WT cells un-transfected. At E9.5 (Figure 4.5 A and C) many neurospheres were formed both from the DT and SC in cultures after Cre-mediated *Sox9* expression, compared to few if any neurospheres formed in all 3 control conditions. Upon dissociation of these primary neurospheres, secondary neurospheres were generated, conferring self-renewal. A similar number of secondary neurospheres formed to primary neurospheres in those cells with Cre-mediated *Sox9* expression (Figure 4.5 B and D), compared to control conditions where few if any neurospheres formed. At E10.5, the number of neurospheres 10 days after electroporation was significantly increased in cells which had undergone Cre-mediated *Sox9* expression (*Z/Sox9* +cre), compared to the 3 control conditions, both from the DT (Figure 4.6 A) and the SC (Figure 4.6 C). Primary neurospheres after Cre-mediated *Sox9* expression from both regions were able to generate secondary neurospheres, confirming self-renewal (Figures 4.6B and 4.6D). From both the DT and SC, the number of secondary

neurospheres which had undergone Cre-mediated *Sox9* up-regulation was significantly greater than the number of neurospheres generated in the 3 control conditions.

**Number of neurospheres formed from the dorsal telencephalon and spinal cord of
E9.5 Z/Sox9 mice compared to controls**

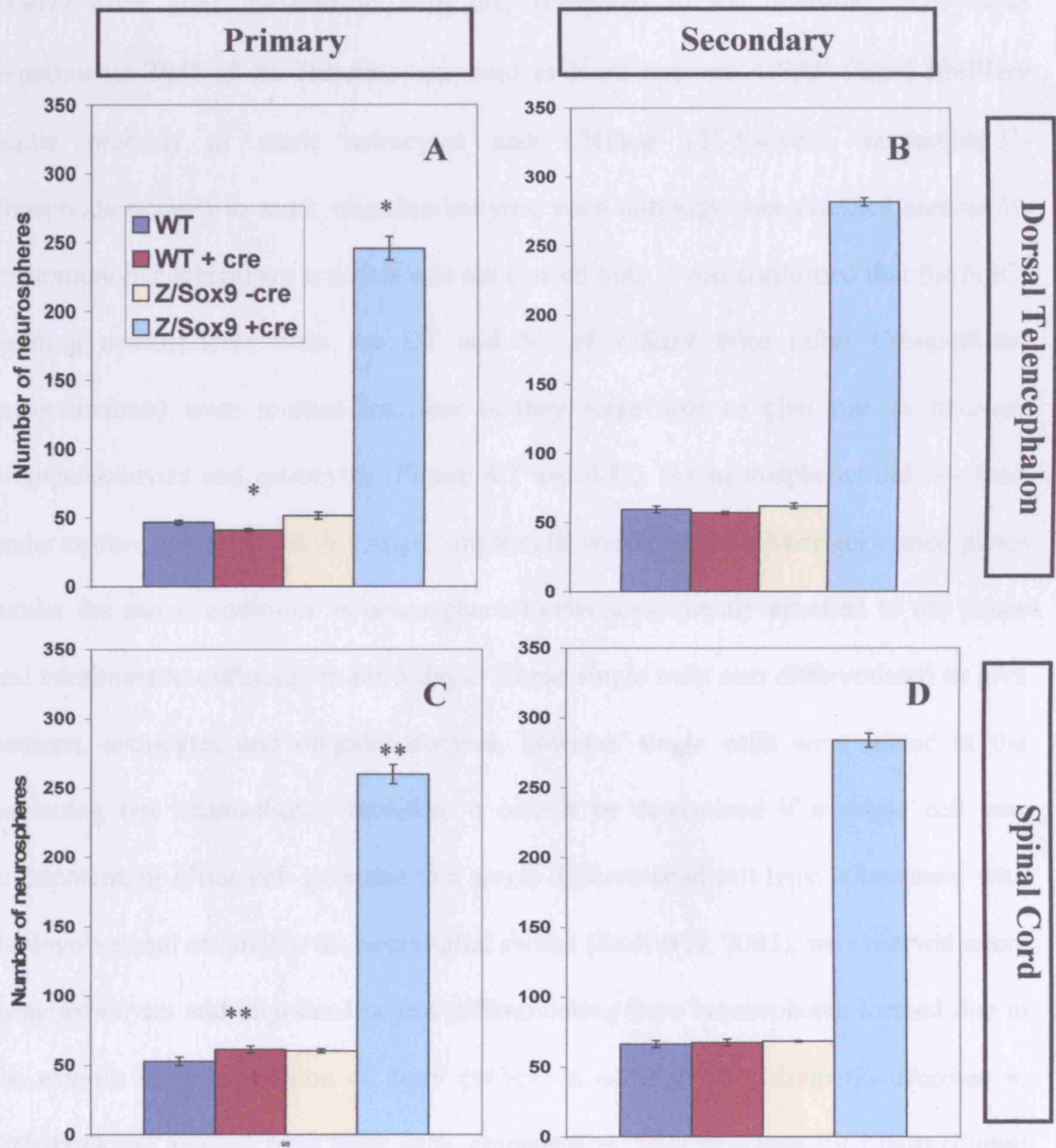


Z/Sox9:



Figure 4.5: At E9.5 the dorsal telencephalon (DT) and spinal cord (SC) are incapable of forming neurospheres *in vitro*. However, using the Z/Sox9 mouse line, whereby upon cre-mediated recombination, Sox9 is expressed, many primary neurospheres were induced from both the DT (A) and SC (C). The difference in neurosphere number between Z/Sox9 (Z/Sox9 + Cre) DT and SC and the controls (Z/Sox9 -Cre, wild type (WT) +Cre and WT) was significant as determined by a Student's t-test *P<0.002; **P<0.002. Upon dissociation of the primary neurospheres generated from the DT and SC of Z/Sox9 +Cre mice, many secondary neurospheres formed (B and D). Few if any secondary neurospheres formed from control cultures. Bars indicate standard error.

**Number of neurospheres formed from the dorsal telencephalon and spinal cord of
E10.5 *Z/Sox9* mice compared to controls**



Z/Sox9:



Figure 4.6: At E10.5 the dorsal telencephalon (DT) and spinal cord (SC) can form a limited number of neurospheres. However, using the *Z/Sox9* mouse line, whereby upon cre-mediated recombination, *Sox9* is expressed, many primary neurospheres were generated from both the DT (A) and SC (C). The difference in neurosphere number between *Z/Sox9* (*Z/Sox9* + Cre) DT and SC and the controls (*Z/Sox9* -Cre, wild type (WT) + Cre and WT) was significant as determined by a Student's t-test * $P < 0.003$; ** $P < 0.003$. Upon dissociation of the primary neurospheres generated from the DT and SC of *Z/Sox9* + Cre mice, many more secondary neurospheres formed (B and D) compared to controls. Bars indicate standard error.

Upon differentiation of the E9.5 neurospheres/cells, immunohistochemistry was carried out to assess the differentiation potential of the cells of the DT and SC from the *Z/Sox9* mice after transfection with cre, compared to the controls. For these experiments TuJ1 (β -III Tubulin) was used to mark neurons, GFAP (Glial fibrillary acidic protein) to mark astrocytes and CNPase (2'-3'-cyclic nucleotide-3'-phosphodiesterase) to mark oligodendrocytes, each antibody was assessed separately, co-immunohistochemistry analysis was not carried out. It was confirmed that the NSCs forming neurospheres from the DT and SC of *Z/Sox9* mice (after Cre-mediated recombination) were multipotent, that is they were able to give rise to neurons, oligodendrocytes and astrocytes (Figure 4.7 and 4.8). As neurospheres did not form under control conditions at this stage, single cells were plated on Matrigel coated plates (under the same conditions as neurospheres) cells subsequently attached to the plates and continued to differentiate for 5 days. These single cells also differentiated to give neurons, astrocytes and oligodendrocytes, however single cells were plated at the beginning not neurospheres, therefore it cannot be determined if a single cell was multipotent, or if one cell gave rise to a single differentiated cell type. Consistent with the involvement of *Sox9* in the neural-glial switch (Stolt et al. 2003), we observed many more astrocytes and oligodendrocytes differentiating from neurospheres formed due to the ectopic early expression of *Sox9* (+Cre), in addition to a dramatic decrease in differentiating neurons from these cells, compared to those wild type E9.5 cells (Figure 4.7 and 4.8). Control cultures (-Cre) from both regions formed many neurons, and very few astrocytes and oligodendrocytes. The percentages of differentiated cell types were calculated by counting 100 Dapi positive cells in 4 separate fields in three different samples, and counting the number of Dapi positive cells that were also positive for the three differentiation markers (figure 4.8), these were assessed separately, and co-immunohistochemistry was not carried out, therefore comparisons should only be

assessed for each differentiated cell type individually between *Z/Sox9* after cre transfection and untransfected cells. In figure 4.8, one possible reason why the percentages of cells marked with the 3 differentiation markers do not add up to 100, could be that a number of the cells co-stain for 2 of the markers used, also the percentage of WT cells do not add up to 100, it is possible that some of these cells could be progenitors, therefore not stain positive for any of the differentiation markers used.

The number of differentiated cell types from E9.5 DT neurosphere cultures with cre-mediated *Sox9* expression (from the *Z/Sox9* mice) are comparable to the numbers of cell types differentiating from E14.5 non-transgenic (WT) DT neurospheres (Figure 4.9 A). In both cases around 20% of cells differentiated into neurons, around 50% into oligodendrocytes, and the majority (~80%) of cells were positive for the astrocyte marker GFAP. The differentiation potential of E9.5 SC neurosphere cultures after Cre-mediated *Sox9* expression are similar to E11.5 SC WT (non-transgenic) neurosphere cultures (Figure 4.9 B), again few neurospheres form and the majority cell type differentiated from both was the astrocyte closely followed by oligodendrocytes. Once more the percentages determined do not total 100%, possibly a number of the cells are positive for more than one differentiation marker used, however co-immunohistochemistry was not carried out therefore this cannot be confirmed. Therefore, comparisons can only be made between the separate differentiation markers generated from neurospheres derived from *Z/Sox9* after transfection with cre, and the non-transgenic CNS tissue, for example the percentage of neurons forming, and not between the differentiated cell types, for example between neurons and astrocytes.

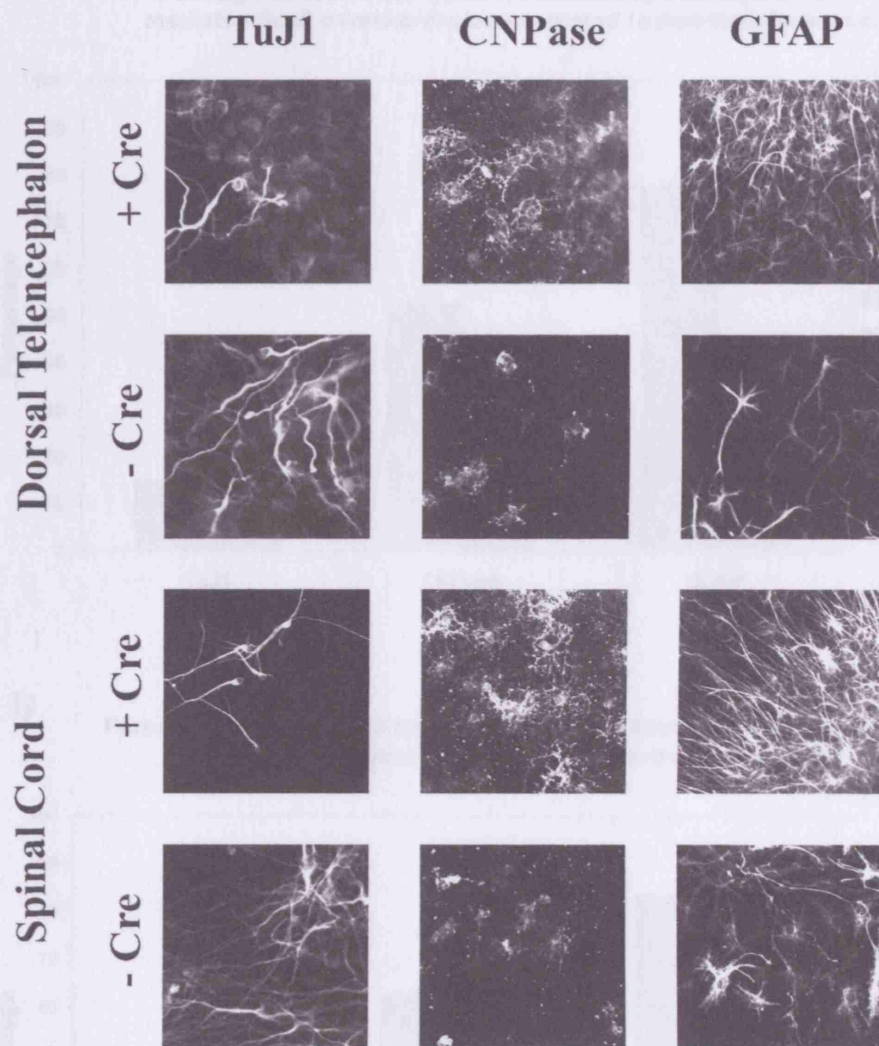


Figure 4.7: E9.5 primary spinal cord (SC) and dorsal telencephalon (DT) neurospheres (or cells in the case of cultures without *Sox9* expression) were allowed to differentiate for 5 days. Differentiation potential was then assessed by immunohistochemistry. Multipotential character of the neurospheres derived from NSCs isolated from *Z/Sox9* SC and DT after cre transfection (+cre) was confirmed by the presence of neurons, oligodendrocytes and astrocytes. Non-transfected (-cre) single cells were able to generate neurons, astrocytes and oligodendrocytes, although multipotentiality cannot be confirmed because it is possible that the single cells were only able to give rise to one differentiated cell type. It was also apparent that neurospheres formed from transfected *Z/Sox9* CNS tissue (+cre) gave rise to more glia and less neurons than untransfected (-cre) cells.

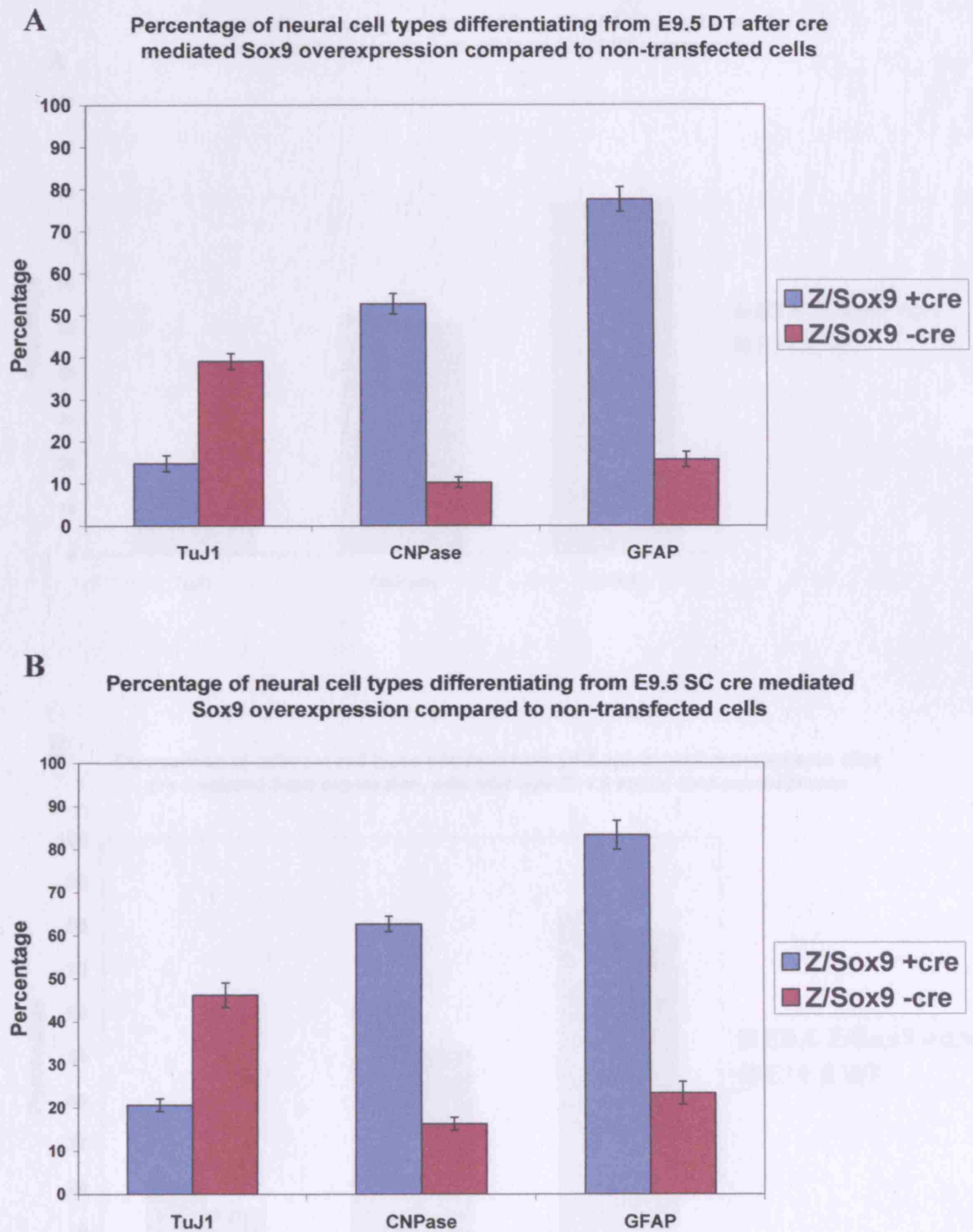
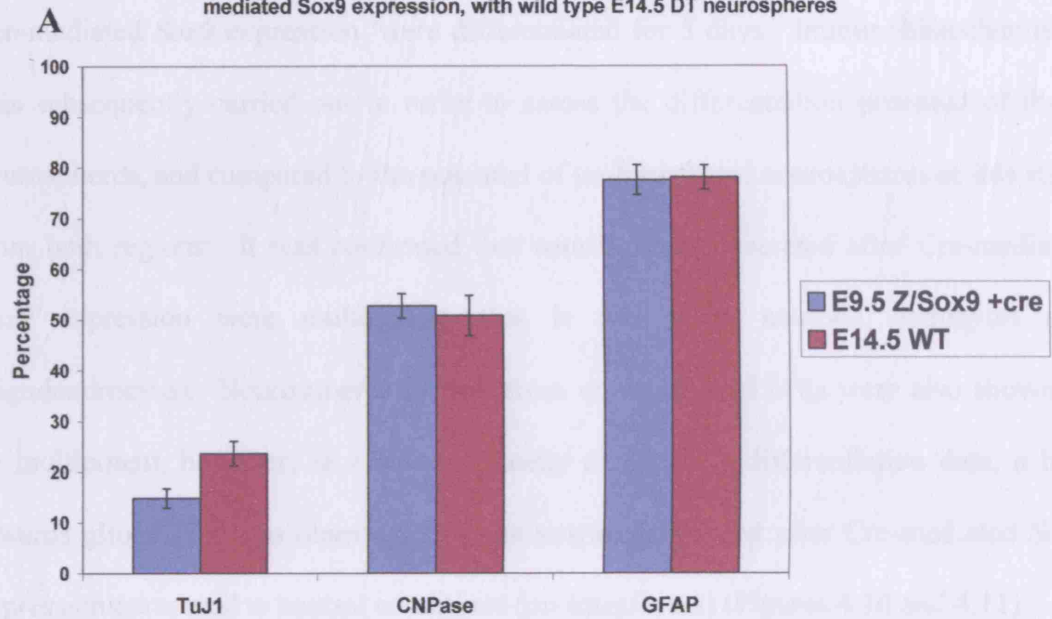


Figure 4.8: Neurospheres or cells from both the cultures with cre-mediated *Sox9* expression (Z/Sox9 +cre) or control cultures (Z/Sox9 -cre) were allowed to differentiate for 5 days. After which time the cell types differentiated were assayed by immunohistochemistry, each antibody was assessed separately, not by co-immunohistochemistry. Four separate fields were analysed in three different samples for each condition. In each field of view, 100 DAPI positive cells were counted, and the percentage of each cell type was then determined. In Z/Sox9 -cre cultures, both dorsal telencephalon (DT) (A) and spinal cord (SC) (B) differentiate into many more neurons and less glia, compared to those from Z/Sox9 +cre cultures. Bars indicate standard error.

Comparison of different cell types obtained from E9.5 DT neurospheres after cre mediated Sox9 expression, with wild type E14.5 DT neurospheres



B Comparison of different cell types obtained from E9.5 spinal cord neurospheres after cre mediated Sox9 expression, with wild type E11.5 spinal cord neurospheres

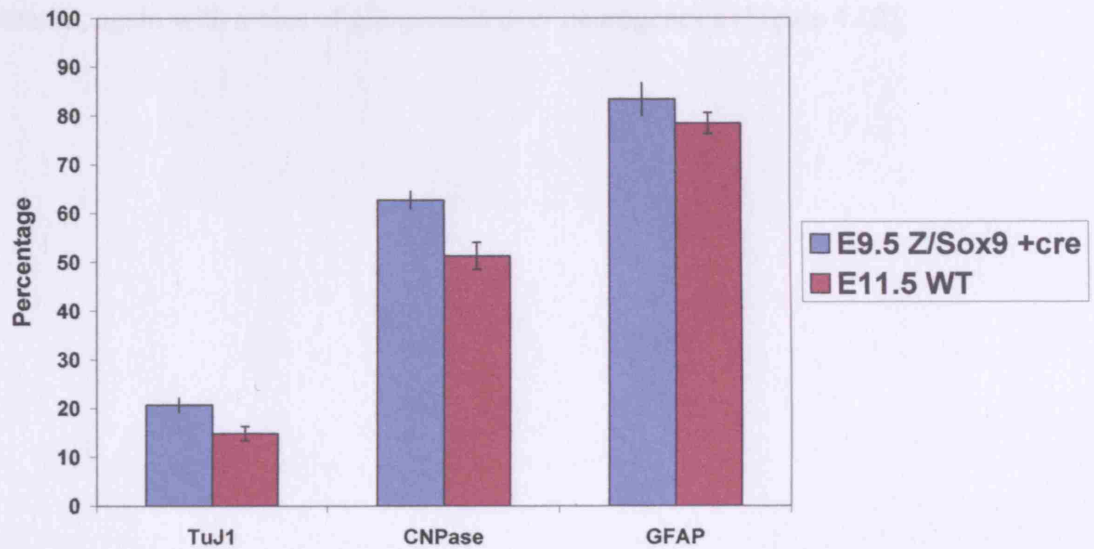


Figure 4.9: The percentages of cell types differentiating from E9.5 dorsal telencephalon (DT) (A) and spinal cord (SC) (B) after cre-mediated expression of *Sox9* (E9.5 Z/Sox9 + cre) are comparable to those from E14.5 DT and E11.5 SC wild type (WT) cultures respectively. Bars indicate standard error.

The E10.5 neurospheres, derived from both the DT and SC of *Z/Sox9* mice, following Cre-mediated *Sox9* expression, were differentiated for 5 days. Immunohistochemistry was subsequently carried out in order to assess the differentiation potential of these neurospheres, and compared to the potential of un-transfected neurospheres at this stage from both regions. It was confirmed that neurospheres generated after Cre-mediated *Sox9* expression were multipotent, that is they form neurons, astrocytes and oligodendrocytes. Neurospheres formed from un-transfected cells were also shown to be multipotent, however, in a similar manner to the E9.5 differentiation data, a bias towards gliogenesis was observed from neurospheres formed after Cre-mediated *Sox9* expression compared to control conditions (un-transfected) (Figures 4.10 and 4.11).

On comparison of the *Sox9* induced E10.5 DT neurosphere differentiation potential to WT E14.5 DT neurospheres, a similar ratio of neurons to glia. E10.5 *Sox9* induced SC neurospheres were comparable to E11.5 WT SC neurospheres in their differentiation potential, again with a bias of gliogenesis over neurogenesis (Figure 4.12).

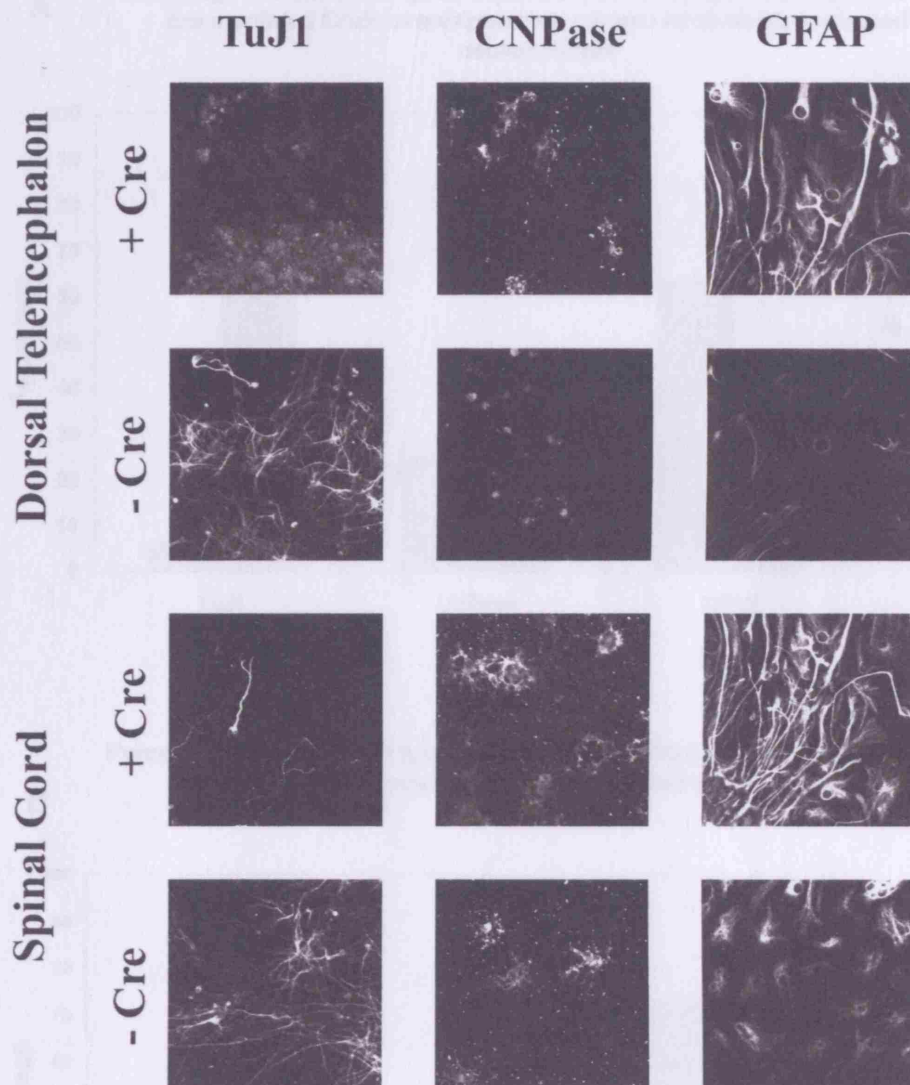
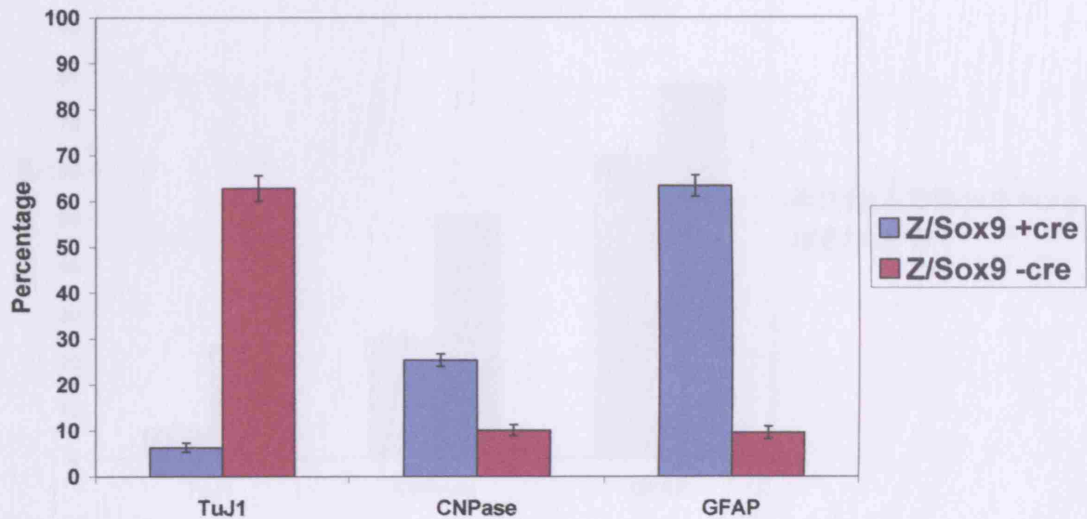


Figure 4.10: E10.5 primary spinal cord (SC) and dorsal telencephalon (DT) neurospheres were allowed to differentiate for 5 days. Differentiation potential was then assessed by immunohistochemistry. Multipotential character of Z/*Sox9* CNS tissue transfected with Cre (+cre), and un-transfected (-cre) was confirmed by the presence of neurons, oligodendrocytes and astrocytes. It was also apparent in both SC and DT cultures that those with ectopic *Sox9* expression (+ Cre) form more glia and less neurons than un-transfected cultures (- Cre).

A Percentage of neural cell types obtained from E10.5 DT neurospheres after cre mediated Sox9 overexpression compared to non-transfected neurospheres



B Percentage of neural cell types obtained from E10.5 SC neurospheres after cre mediated Sox9 overexpression compared to non-transfected neurospheres

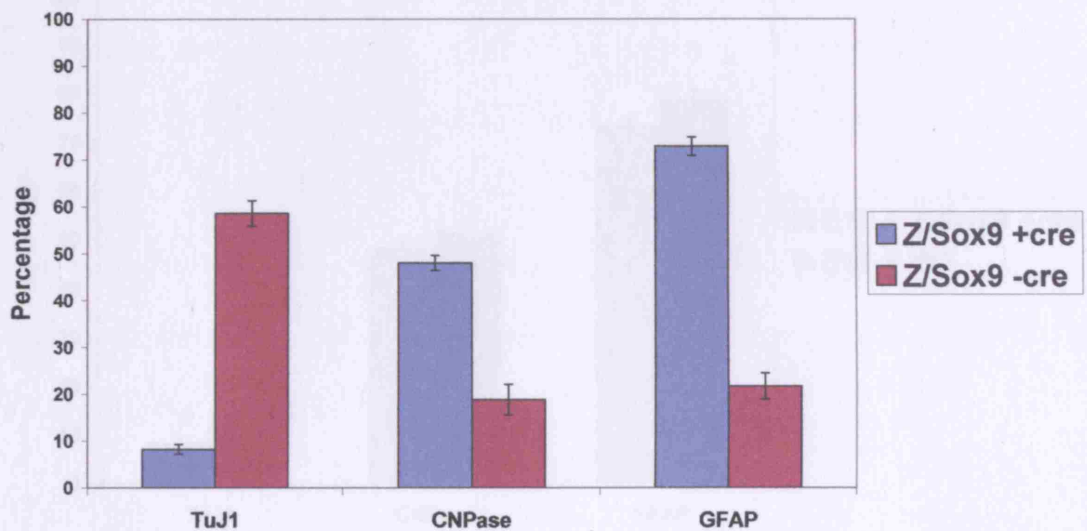
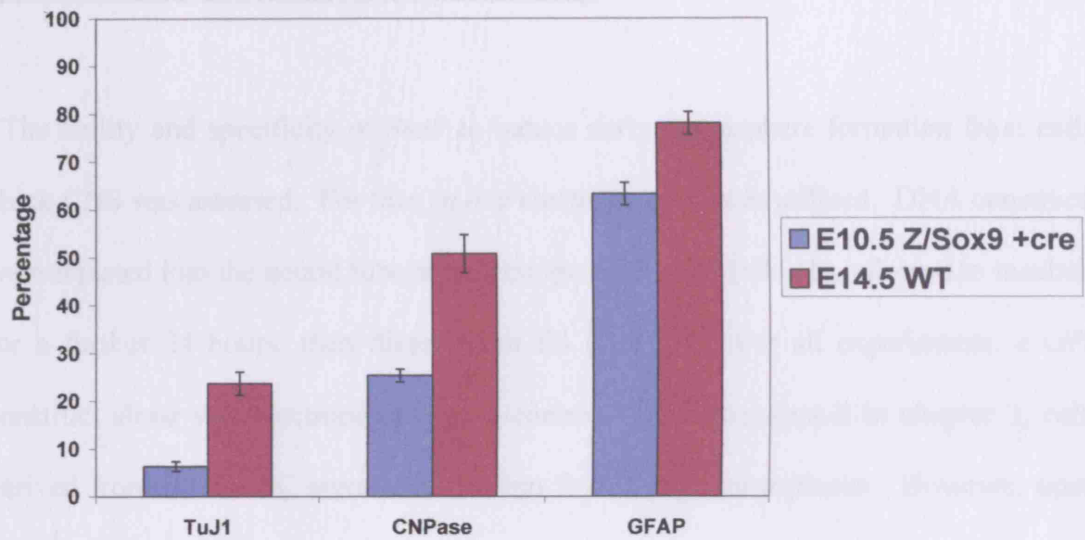


Figure 4.11: Neurospheres derived from E10.5 dorsal telencephalon (DT) and spinal cord (SC) neurospheres were allowed to differentiate for 5 days. Differentiation potential was then assessed by immunohistochemistry. Multipotential character was confirmed by the presence of neurons, oligodendrocytes and astrocytes obtained from both Z/Sox9 +cre and non-transfected neurospheres. It was also apparent in both DT (A) and SC (B) cultures that those with ectopic Sox9 (Z/Sox9 +cre) expression form more glia and less neurons than non-transfected cultures (Z/Sox9 -cre). Bars indicate standard error.

A Comparison of neural cell types obtained from E10.5 DT neurospheres after cre mediated Sox9 expression, versus wild type E14.5 DT



B Comparison of neural cell types obtained from E10.5 SC neurospheres after cre mediated Sox9 expression, versus wild type E11.5 SC neurospheres

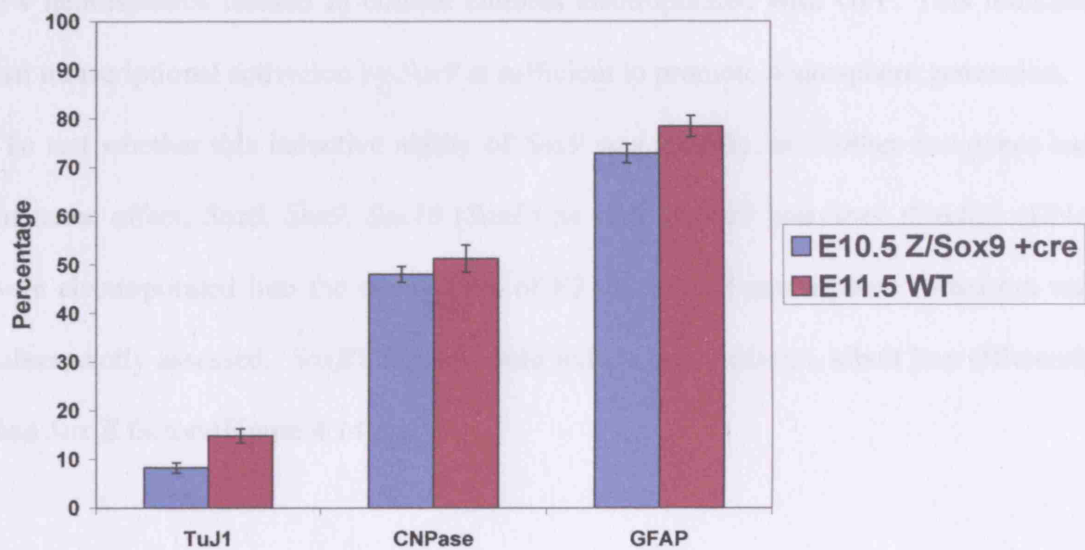


Figure 4.12: One hundred DAPI-positive cells were counted in four fields of three sections per condition, the percentage of each cell type was then calculated. The percentages of neurons, oligodendrocytes and astrocytes formed from E10.5 dorsal telencephalon (DT) neurospheres (A) and spinal cord (SC) (B) after cre-mediated Sox9 expression (E10.5 Z/Sox9 +cre) were similar to wild type (WT) E14.5 DT and E11.5 SC respectively. Bars indicate standard error.

4:4:3 Ectopic *Sox9* is able to induce neurosphere formation from early chick CNS, and this depends on transcriptional activation

The ability and specificity of *Sox9* to induce early neurosphere formation from early chick CNS was assessed. For this, *in ovo* electroporation was utilized. DNA constructs were injected into the neural tube and electroporated at E3 (HH 18), allowed to incubate for a further 24 hours, then dissected at E4 (HH 23). For all experiments, a GFP construct alone was electroporated as a control. As demonstrated in chapter 3, cells derived from the E4 SC were able to form few if any neurospheres. However, upon transfection of pCAGGS-*Sox9*-IRES-nls-GFP (*Sox9*) (Cheung and Briscoe, 2003) or a *Sox9* construct with a heterologous VP16 transactivator domain, *Sox9*-VP16 (Cheung et al. 2005) (VP16S9), many neurospheres were induced (Figure 4.13), compared to very few neurospheres formed in control cultures electroporated with GFP. This indicates that transcriptional activation by *Sox9* is sufficient to promote neurosphere generation.

To test whether this inductive ability of *Sox9* was specific, or if other *Sox* genes had the same effect, *Sox8*, *Sox9*, *Sox10* (*SoxE*) as well as *Sox1* and *Sox2* (*SoxB1*) cDNA were electroporated into the neural tube of E3 chicks and neurosphere formation was subsequently assessed. *SoxB1* factors could induce neurospheres, albeit less efficiently than *Sox E* factors (Figure 4.14).

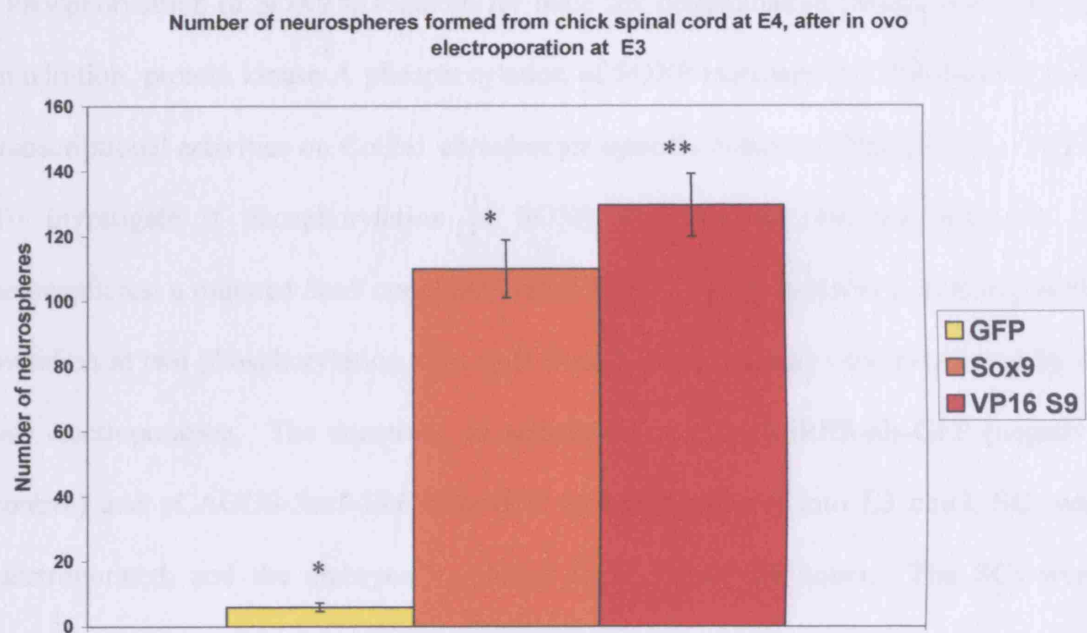


Figure 4.13: E3 chick neural tubes were electroporated with a control construct, pCAGGS-IRES-nls-GFP (GFP), or pCAGGS-*Sox9*-IRES-nls-GFP (*Sox9*) or a version of *Sox9* containing a VP16 transactivator domain, *Sox9*-VP16 (VP16S9). Very few neurospheres formed in GFP control cultures, both *Sox9* and VP16-*Sox9* were able to induce neurosphere generation. Bars indicate standard error. * $P < 0.003$ ** $P < 0.002$.

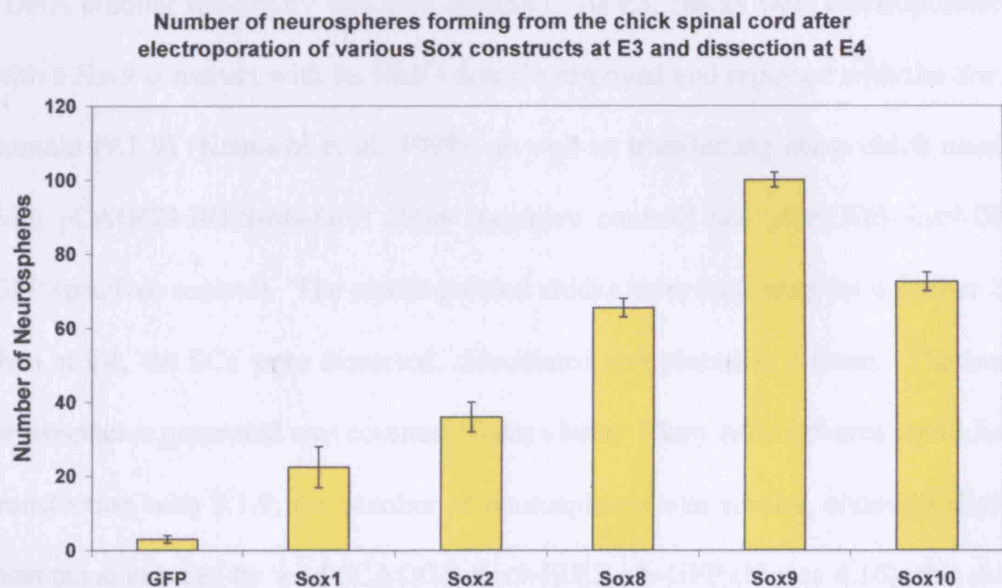


Figure 4.14: E3 chick neural tubes were electroporated with GFP (control), *SoxB1* factors *Sox1* and *Sox2*, and *SoxE* group factors *Sox8*, *Sox9* and *Sox10*. Both *SoxB1* and *SoxE* factors were able to induce neurosphere generation, however, *SoxE* factors produced many more spheres. The electroporation with *Sox9* generated the most neurospheres, producing more than double the number of spheres formed by *Sox1* or *Sox2*. Bars indicate standard error.

Phosphorylation of SOX9 is required for male sex determination (Malki et al., 2005), in addition, protein kinase A phosphorylation of SOX9 increases its DNA binding and transcriptional activities on Col2a1 chondrocyte-specific enhancer (Huang et al., 2000). To investigate if phosphorylation of SOX9 was required for the induction of neurospheres, a mutated *Sox9* construct, S64A S181A, which contains a double alanine mutation at two phosphorylation sites (gift from Martin Cheung) was transfected by *in ovo* electroporation. The construct, in addition to pCAGGS-IRES-nls-GFP (negative control) and pCAGGS-*Sox9*-IRES-nls-GFP (positive control) into E3 chick SC, was electroporated, and the embryos incubated for a further 24 hours. The SCs were dissected, dissociated to single cells and transferred to culture, neurosphere formation was analysed 10 days later. S64A S181A could form a similar number of neurospheres to wild type *Sox9* (Figure 4.15), therefore it seems that phosphorylation is not necessary for neurosphere formation.

DNA binding specificity was then assessed. At E3, chicks were electroporated *in ovo* with a *Sox9* construct with its HMG domain removed and replaced with the *Sox1* HMG domain (9.1.9) (Kamachi et al. 1999), as well as transfecting some chick neural tubes with pCAGGS-IRES-nls-GFP alone (negative control) and pCAGGS-*Sox9*-IRES-nls-GFP (positive control). The electroporated chicks were incubated for a further 24 hours, then at E4, the SCs were dissected, dissociated and placed in culture. The number of neurospheres generated was counted 10 days later. Many neurospheres could form after transfection with 9.1.9, the number of neurospheres was similar, although slightly less than those induced by wild pCAGGS-*Sox9*-IRES-nls-GFP (Figure 4.16), this difference was significant as determined by a Student's t-test, $P < 0.01$. This indicates that although most of the specificity resides outside the DNA binding domain, the difference in the sequence of the HMG box (between *Sox1* and *Sox9*) has some specificity although limited, and appears to play a minor role in neurosphere generation.

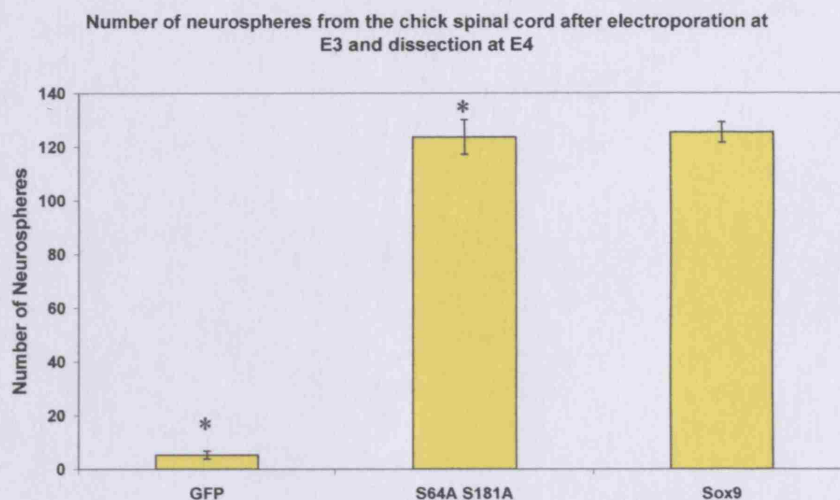


Figure 4.15: E3 chick spinal cords were electroporated with a *Sox9* mutated construct, at E4, spinal cords were dissected and transferred to tissue culture and the number of neurospheres formed after 10 days was counted, and compared to the number generated by GFP alone and wild type *Sox9*. S64A S181A is a double mutation at phosphorylation sites, and was able to induce many neurospheres comparable to wild type *Sox9*. Few if any neurospheres could form from spinal cord electroporated with GFP alone. The difference in number in number of neurospheres between GFP and S64A S181A was determined to be statistically significant by a Student's t-test, * $P < 0.001$. Bars indicate standard error.

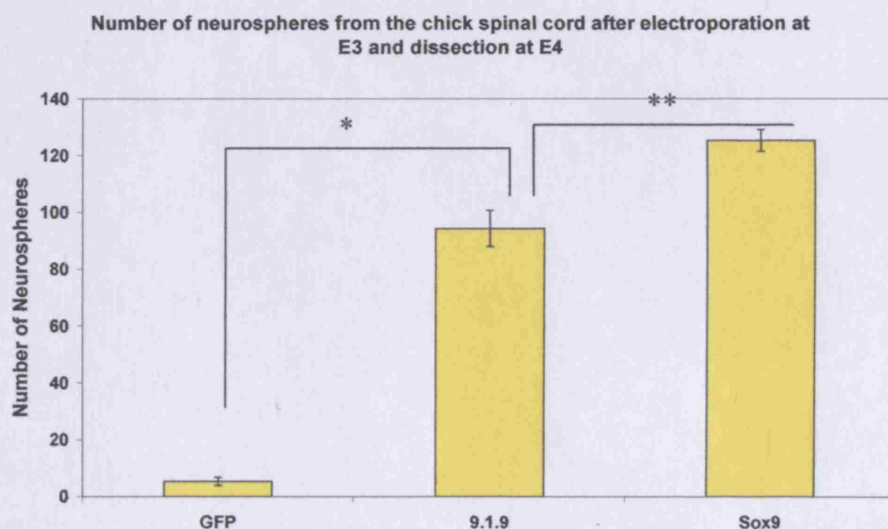


Figure 4.16: At E3, cells from the chick spinal cord (SC) are unable to generate neurospheres. Chick SCs were electroporated at E3, dissected at E4 and neurosphere forming ability assessed after 10 days in culture. The electroporation *in ovo* of pCAGGS-*Sox9*-IRES-nls-GFP (*Sox9*) is able to induce neurosphere formation. When the HMG domain of *Sox9* was replaced with the HMG domain of *Sox1* (9.1.9), many neurospheres were also induced, and the number was statistically significant from GFP control transfected cultures as determined by a Student's t-test, * $P < 0.002$. The difference between the number of neurospheres formed from 9.1.9 and *Sox9* transfected cells is significant ** $P < 0.01$, therefore most of the specificity resides outside the DNA binding domain. Bars indicate standard error.

4:4:4 Transfection of *Sox9* is able to increase neurosphere generation in adult subventricular zone cultures

Several adult CNS stem cell niches have been identified, among these the subventricular zone (SVZ), contains cells well established as being capable of generating neurospheres. Therefore, it was asked if transfecting *Sox9* into cells of this region was able to increase neurosphere formation. SVZ cells from 8week old mice were electroporated with either pCAGGS-*Sox9*-IRES-nls-GFP or pCAGGS-IRES-nls-GFP (control) and were allowed to form primary neurospheres. Initially, it was noticed that cultures electroporated with *Sox9* generated neurospheres at a much faster rate (after just 4 days) compared to control cultures (neurospheres were not apparent until ~7days) (Figure 4.17). The speed at which *Sox9* transfected adult cultures formed neurospheres was comparable to normal embryonic cultures, where neurospheres are observed after 2-3days. After 10 days in culture, more than double the number of primary neurospheres in *Sox9* transfected cultures were counted compared to control GFP transfected cultures (Figure 4.18). Significantly, there was an increase in secondary neurosphere generation. More than double the number of secondary neurospheres derived from *Sox9* transfected cultures compared to control cultures. This provides evidence that NSCs capable of self-renewal are resident in the SVZ, and self-renewal is increased by transfection with *Sox9*. In this instance, the initial transient transfection of *Sox9* was able to induce a long lasting effect on NSC number.

To assess the differentiation potential of adult neurospheres after transient transfection with *Sox9* compared with control GFP transfected cultures, primary neurospheres were allowed to differentiate for 5 days, then assessed by immunohistochemistry. First, it was confirmed that all the neurospheres formed generated all three main cell types of the CNS (Figure 4.19). In agreement with the known role of *Sox9* in gliogenesis, many

more glia and less neurons were obtained from SVZ neurospheres electroporated with *Sox9* compared to control cultures (Figure 4.19).

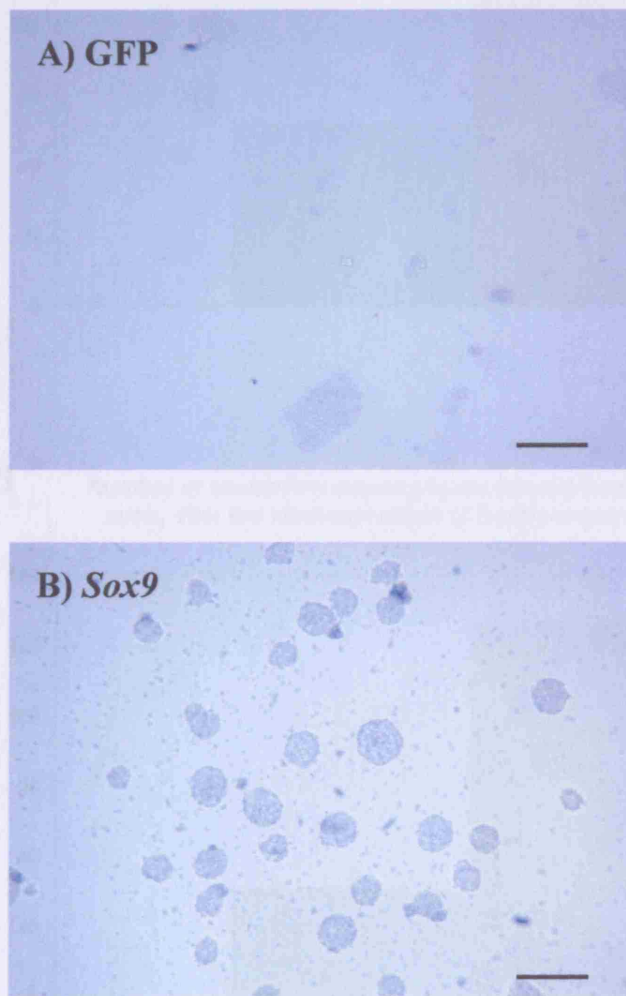


Figure 4.17: Cells from the adult subventricular zone were electroporated with either the control construct, pCAGGS-IRES-nls-GFP (GFP) or pCAGGS-*Sox9*-IRES-nls-GFP (*Sox9*). After just 4days in culture neurosphere were already apparent in *Sox9* electroporated cultures (B), no neurospheres were visible at this stage in the control GFP transfected cultures (A). Scale bars = 50µm.

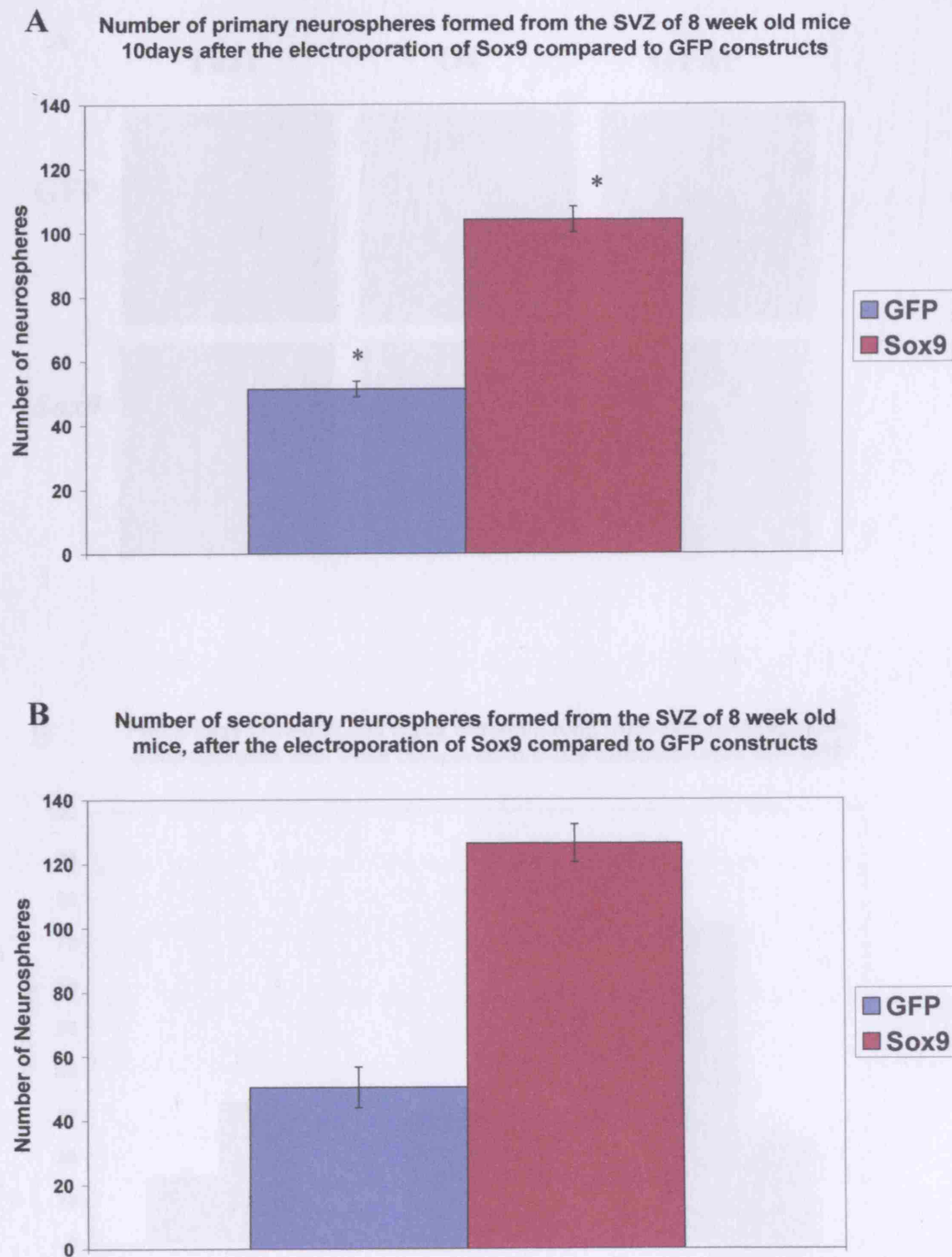


Figure 4.18: Cells from the adult (8 week old) subventricular zone (SVZ) were electroporated with either pCAGGS-*Sox9*-IRES-nls-GFP (*Sox9*) or pCAGGS-IRES-nls-GFP (GFP) expression constructs. After 10 days in culture, the numbers of primary neurospheres were counted. More than double the number of neurospheres were present in *Sox9* transfected cultures compared to control GFP transfected cultures, this was determined to be statistically significant by a Student's t-test, * $P < 0.005$ (A). Upon dissociation of these primary neurospheres, the number of secondary spheres was analysed. More than double the number of secondary neurospheres in *Sox9* transfected cultures compared to control cultures were observed (B). Bars indicate standard error.

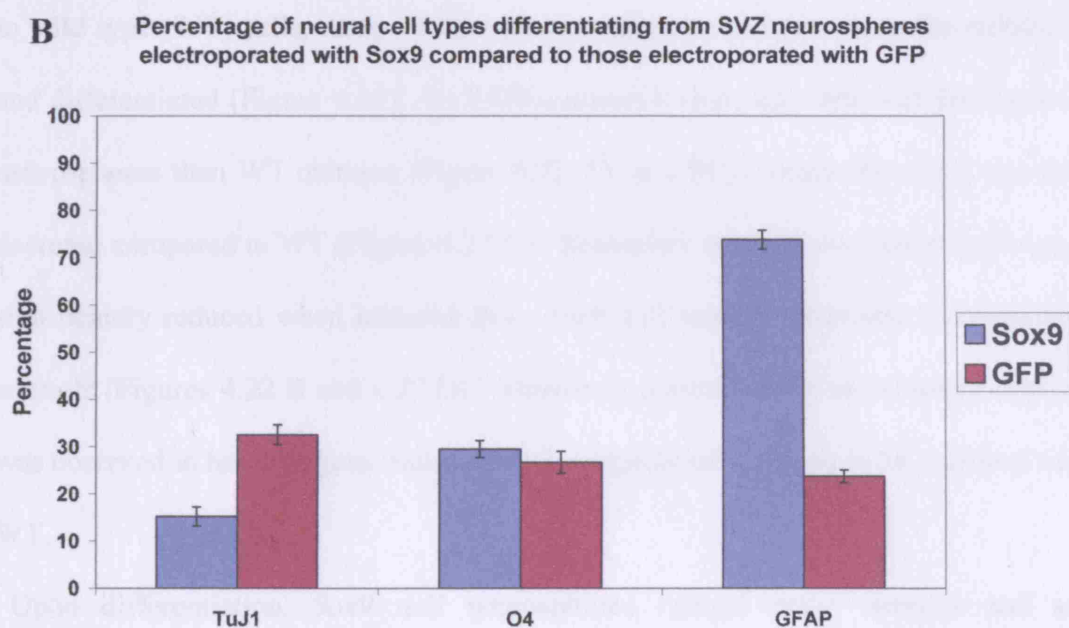
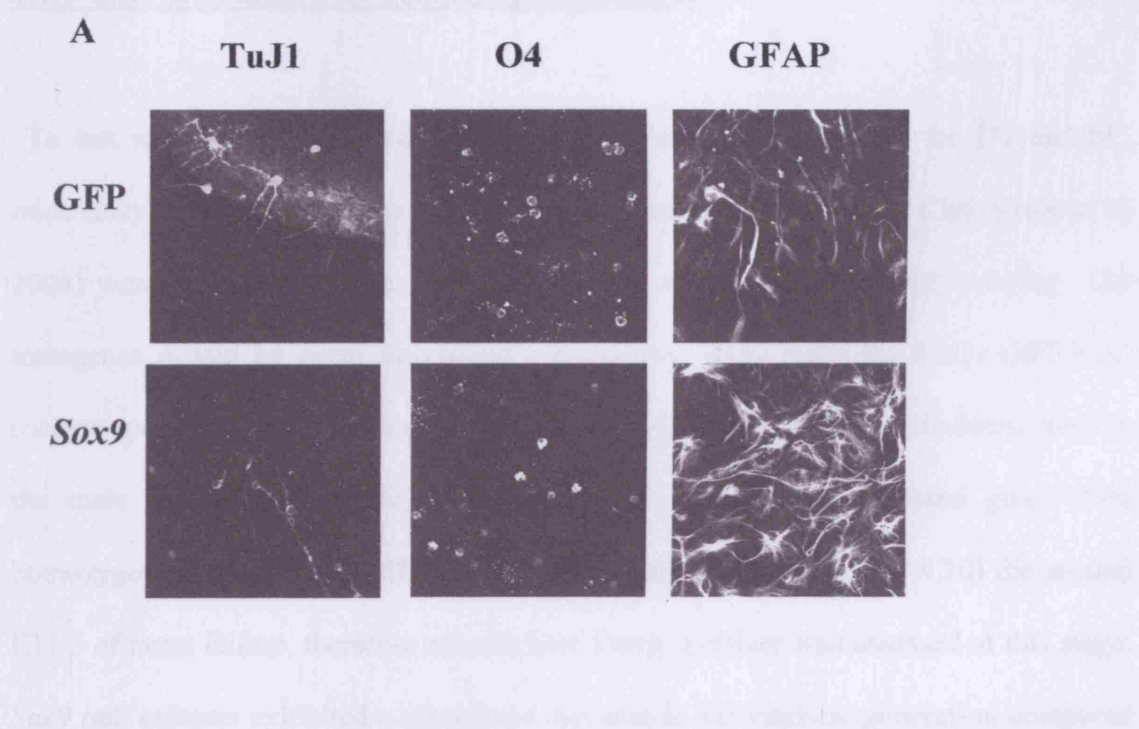


Figure 4.19: Neurospheres derived from cells of the adult subventricular zone (SVZ) were able to give rise to neurons, astrocytes and oligodendrocytes (A). The percentages of each neural cell type generated were calculated as before. Around half the number of neurons (TuJ1 positive) formed in pCAGGS-*Sox9*-IRES-nls (Sox9) transfected cultures compared to control, pCAGGS-IRES-nls-GFP transfected (GFP), and more than double the number of astrocytes (GFAP) formed. The number of oligodendrocytes (O4) remained unchanged (B). Bars indicate standard error.

4:4:5 Sox9 is necessary for neurosphere formation

To test whether *Sox9* is necessary for neurosphere formation from the DT and SC, mice carrying a conditional mutation of *Sox9* mouse line (Flox-*Sox9*, Chaboissier et al. 2004) were analysed. These “Flox-*Sox9*” mice were bred with mice carrying Cre transgenes driven by germ line-specific promoters; Zone pellucida 3 Cre (ZP3-Cre) (oocyte specific) and Protamine 1 Cre (Prm1-Cre) (spermatid specific) to delete *Sox9* in the male and female germline (Akiyama et al., 2002), these crossed give ~25% homozygous null embryos. The resulting *Sox9* null embryos (Figure 4.20) die around E11.5 of heart failure, therefore neurosphere forming ability was assessed at this stage. *Sox9* null cultures exhibited a significant decrease in neurosphere generation compared to wild type (WT) cells, many of the cells in null cultures attached to the culture dish and differentiated (Figure 4.21). Null DT cultures formed less than half the number of neurospheres than WT cultures (Figure 4.22 A), and SC cultures showed a seven-fold decrease compared to WT (Figure 4.22 C). Secondary neurosphere generation was also significantly reduced when initiated from *Sox9* null mutant neurospheres compared to controls (Figures 4.22 B and 4.22 D). Moreover, a reduction in neurosphere formation was observed in heterozygous mutants, with a significant decrease in SC cultures versus WT.

Upon differentiation, *Sox9* null neurospheres formed many neurons and some astrocytes, but few if any oligodendrocytes (Figure 4.23). When compared to WT neurospheres, *Sox9* null neurospheres were able to differentiate into many more neurons and many less oligodendrocytes and astrocytes (once again this was consistent with the role for *Sox9* in gliogenesis). Moreover, it was also observed that many more *Sox10* positive cells were present in *Sox9* null cultures compared to WT (Figure 4.24). Which

raises the possibility that in the absence of *Sox9*, the only cells that can form neurospheres, are those expressing SOX10, possibly indicating a compensatory role.

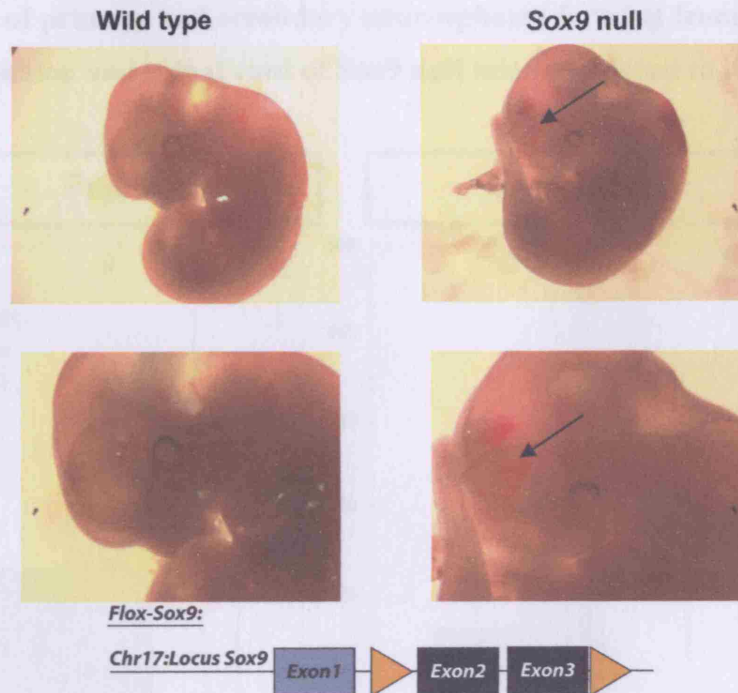


Figure 4.20: Embryos lacking *Sox9* (*Sox9* null) were generated by germline Cre-mediated recombination in the germline, shown here at E11.5, were compared with wild type litter mates, their dorsal telencephalon was noticeably truncated (indicated by the arrow), and irregular in shape. There was no obvious phenotype in the spinal cord.

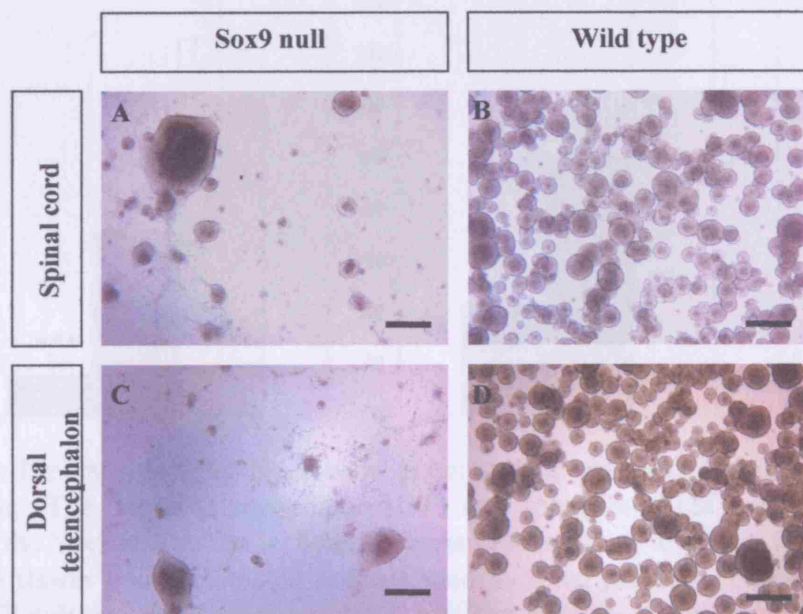


Figure 4.21: Null *Sox9* embryos were generated by Cre-mediated recombination in the germline, and the spinal cord and dorsal telencephalon dissected at E11.5. The tissue was dissociated and cultured to assess neurosphere forming ability. Following 10 days in culture, the number of primary neurospheres was counted. The *Sox9* null tissue from both regions formed a small number of neurospheres, and many of the cells attached to the tissue culture dish and differentiated (A and C). Cells from both regions of wild type litter mates formed many, floating neurospheres (B and D). Scale bars = 50µm.

Number of primary and secondary neurospheres forming from the dorsal telencephalon and spinal cord of Sox9 null mice compared to litter mates

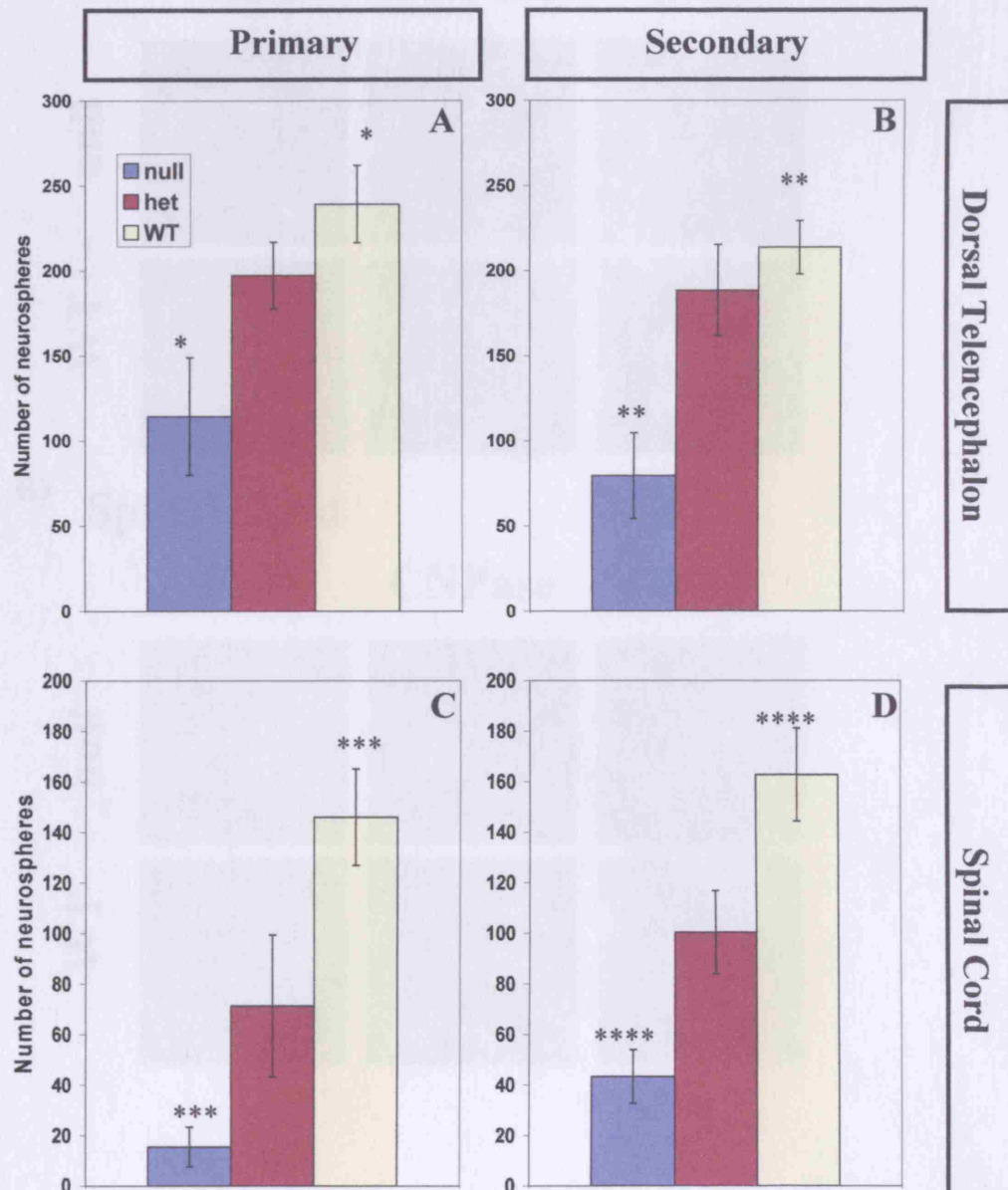
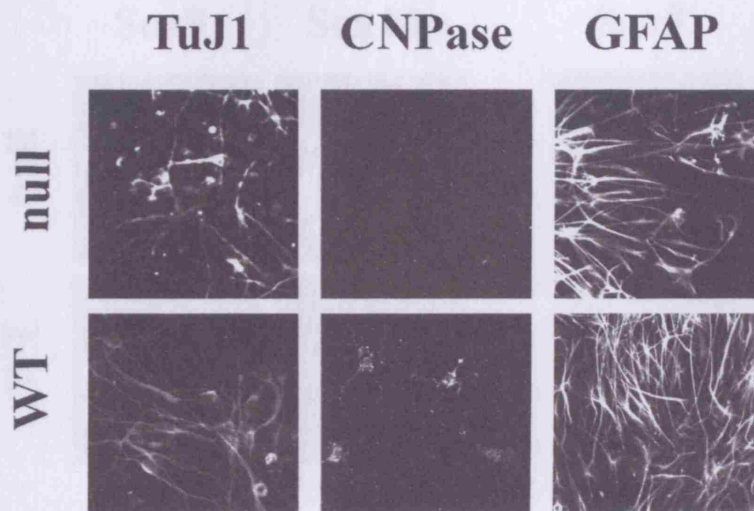


Figure 4.22: Embryos lacking *Sox9* were generated by cre-mediated recombination in the germline. The dorsal telencephalon (DT) and spinal cord (SC) were dissected at E11.5 from the *Sox9* null mutants, heterozygous mutants (het) and wild type (WT) litter mates. The tissue was dissociated and allowed to form primary neurospheres for 10 days. In DT cultures there were less than half the number of neurospheres generated from *Sox9* null cultures versus WT. When these primary neurospheres were dissociated to form secondary neurospheres much less than half the number of secondary neurospheres formed from null cultures. In SC cultures there was a seven-fold decrease in primary neurosphere generation versus WT, also heterozygous mutants could only generate half the number of WT primary neurospheres. A four-fold decrease was found in secondary neurosphere formation in *Sox9* null cultures compared to WT. * $P < 0.008$, ** $P < 0.003$, *** $P < 0.001$, **** $P < 0.003$ as determined by a Student's *t*-test. Bars indicate standard error.

A) Dorsal Telencephalon



B) Spinal Cord

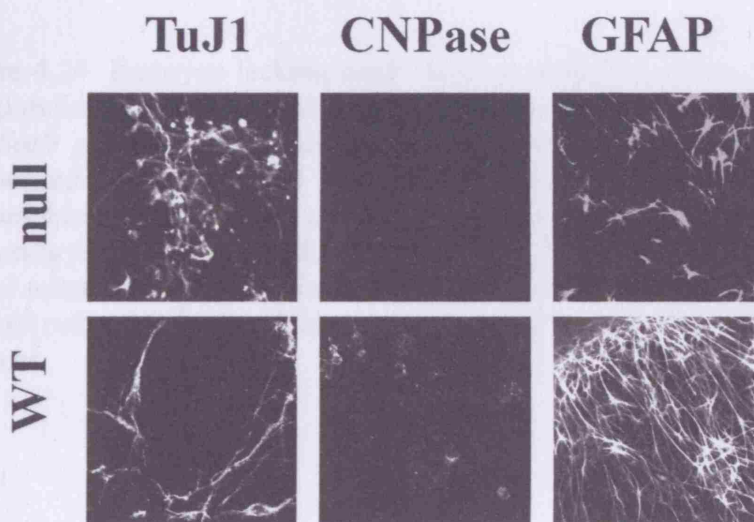


Figure 4.23: Embryos lacking *Sox9* were generated by cre-mediated recombination in the germline. The dorsal telencephalon (A) and spinal cord (B) were dissected at E11.5 from the *Sox9* null mutants (null), and wild type (WT) litter mates. The tissue was dissociated and allowed to form primary neurospheres for 10 days. These primary neurospheres were differentiated for 5 days. Subsequent immunohistochemistry revealed null cultures formed many more neurons (TuJ1), less astrocytes (GFAP) and few if any oligodendrocytes (CNPase) compared to WT neurospheres at this stage.